THE THERMAL LOSS OF VITAMIN B_1 ON STORAGE OF FOODSTUFFS

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Considerable attention has been directed to the loss of vitamin B_1 potency from various foodstuffs, both fresh and processed, which have been stored for any length of time. However, the criticism which may be made of the work at processing temperatures may also be made of the published work on losses in storage, viz., that there is no attempt to correlate results obtained for destruction of this vitamin in different products.

The present author has used the determination of the velocity coefficient for the thermal destruction of aneurin and cocarboxylase to study the effect of a number of factors on this destruction. This paper proposes a similar approach to studies of vitamin B_1[1] losses on storage of foodstuffs where such losses are thermal but takes no account of losses due to enzymic or microbiological activity which may occur in, for example, unprocessed fruit, vegetables and meat which are frequently held in cold storage.

The thermal destruction of vitamin B_1 is a first order reaction for which the velocity coefficient, k, is given by the well-known equation

$$k = \frac{1}{t} ln \frac{a}{a-x}$$

where "a" is the initial concentration of the vitamin and "x" the concentration remaining after time "t" which is measured in minutes. The value of k varies between wide limits according to pH, temperature and other usually less important factors, but, in foodstuffs, will usually fall in the range 0·001 to 0·05 at 100° C. In storage experiments where time intervals required for significant changes in "x" to occur may be weeks or even months, k becomes microscopically small. It is, therefore, proposed that a new constant k_s (S = Storage) be used to denote the velocity coefficient for the thermal destruction of vitamin B_1 on storage. k_s is calculated in exactly the same way as k but it is measured in weeks rather than minutes. k_s is thus related to k by the factor $\frac{1}{10080}$ (1 week = 10080 minutes) and, having regard to the experimental errors involved, is virtually equal to $k \times 10^4$.

[1] For the purposes of this paper the term "vitamin B_1" is considered to include aneurin, cocarboxylase and protein-bound aneurin.

This constant is used in the work recorded below dealing with loss of vitamin B_1 from cheese. In addition, data obtained by earlier authors in studies on losses of vitamin B_1 from various foodstuffs on storage have, where possible, been examined and k_s calculated. The results obtained show that k_s provides a simple quantitative basis for the comparison of the behaviour of vitamin B_1 in different foodstuffs under different conditions.

EXPERIMENTAL.

Samples were held in incubators at 21°, 37°, 49° and 65° C. and withdrawn at the time intervals specified. Eight-ounce cartons of processed cheese were stored at the two lower temperatures for six months, samples being withdrawn for assay at intervals of one month. For the tests at 49° C., molten cheese from the processing kettle was cast in half Petri dishes and contact sealed with a glass plate. These samples were incubated and withdrawn at intervals of 3–4 days and held in the refrigerator until the conclusion of the experiment when all were assayed together.

The method of preparing the cheese for assay was that suggested by Houston, Kon and Thompson (1940) and involved incubation for two days with pepsin followed by 6 hours with takadiastase before washing the acidified solution with water-saturated butyl alcohol and proceeding to the final thiochrome assay.

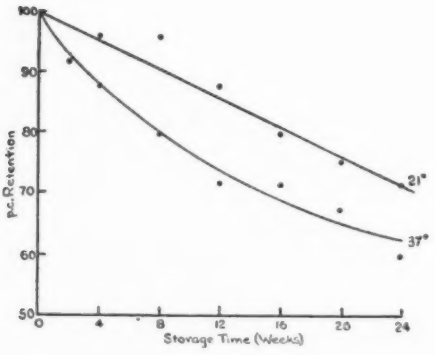

Fig. 1.

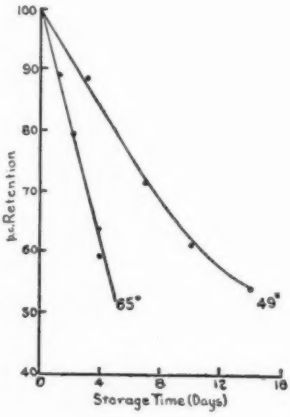

Fig. 2.

Fig. 1. Retention of Vitamin B_1 on storage of processed cheese at 21° and 37° C.
Fig. 2. Retention of Vitamin B_1 on storage of processed cheese at 49° and 65° C.

In Figs. 1 and 2, percentage retention of vitamin B_1 is plotted against storage time. If the logarithm of the percentage retention is plotted against time, k_s may be calculated from the slope of the resulting straight line. Values of k_s obtained for the temperatures studied are found in Table 1.

TABLE 1.

k_s, the velocity coefficient, for the rate of destruction of Vitamin B_1 in stored cheese.

Storage temperature	k_s
21° C.	0·014
37° C.	0·069
49° C.	0·30
65° C.	0·87

Fig. 3 demonstrates the temperature effect and is the curve for the Arrhenius equation, $\ln k_s = I - E/RT$. Log k_s is plotted against $1/T$ (T is measured in degrees absolute), I, E and R being constant. A straight line results, the deviations undoubtedly being due to experimental error and also to the fact that two different samples of cheese were used. In cheese there is free aneurin, cocarboxylase and protein bound aneurin (Dearden *et al.*, 1945; Evans *et al.*, 1946) and k_s must therefore represent the average of the rates of destruction of the three forms of vitamin B_1.

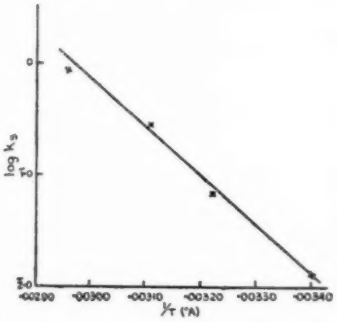

Fig. 3.

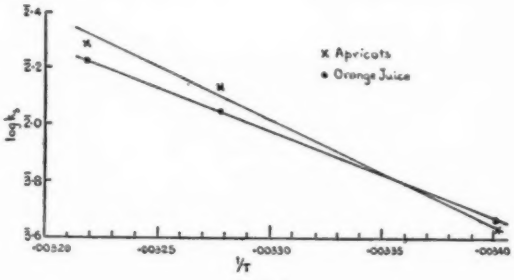

Fig. 4.

Fig. 3. Arrhenius Curve for B_1 destruction on storage of cheese.

Fig. 4. log $k_s/1/T$ Curves for aneurin destruction on storage of canned apricots and orange juice. Calculated from results of Brenner, Wodicka and Dunlop (1948).

DISCUSSION.

When work published in the literature is examined, it is found that it is not possible to calculate k_s in some cases as the experimental results are presented as graphs from which the points cannot be transposed with any precision. Such work is that of Guerrant, Vavich and Dutcher (1945) on the loss of vitamin B_1 from tomato juice, lima beans and yellow corn on storage at temperatures of 30°, 42°, 85° and 110° F. for a year. In fact, there is no significant loss at the two lower temperatures in that time, but the graphs indicate that values for k_s could easily be obtained at 85° and 100° F. Similarly, Guerrant, Fardig, Vavich and Ellenberger (1948) have studied losses from asparagus, spinach, peas, beans and corn on storage up to two years at 50°, 65° and 80° F. The same general results were obtained and there is little doubt that k_s could be determined.

The results of Clifcorn and Heberlein (1944) for storage tests at room temperature on asparagus, lima beans, corn and peas do not permit the calculation of k_s; some are in any case contradictory. The same holds for the studies of Moschette, Hinman and Halliday (1947) on storage losses of aneurin from canned fruits and fruit juices. These authors, too, found losses at 80° F. and over but not at 65° F. or less. Again it was not possible to make any calculation of k_s.

However, a recent paper of Brenner, Wodicka and Dunlop (1948) has presented results which permit a kinetic treatment. These authors studied the loss of aneurin, *inter alia*, in certain canned fruit and vegetable products. Their results were presented as graphs showing retention plotted on a logarithmic scale (in fact, log $(a-x)$) against the time of storage. By photographing these graphs and enlarging them to twice their size, it has been possible to calculate k_s, with a reasonable degree of assurance, from the slopes of a number of the curves. The values obtained are shown in Table 2.

TABLE 2.

The velocity coefficient (k_s) for the destruction of aneurin on storage of some canned fruit and vegetable products.

(Calculated from the results of Brenner, Wodicka and Dunlop (1948)).

Product	Storage temperature	k_s
Peas	100° F.	0·013
	90° F.	0·0065
Lima Beans	100° F.	0·011
	90° F.	0·0097
Orange Juice	100° F.	0·017
	90° F.	0·011
	70° F.	0·0047
Tomato Juice	100° F.	0·0096
	90° F.	0·0040
Apricots	100° F.	0·019
	90° F.	0·014
	70° F.	0·0043

TABLE 3.

The velocity coefficient (k_s) for the destruction of aneurin on storage of canned pork.

A. Calculated from the results of Rice and Robinson (1944).

Storage temperature	k_s
80° F. (26·7° C.)	0·012
99° F. (37·2° C.)	0·029
120° F. (48·9° C.)	0·13
145° F. (62·8° C.)	0·37

B. Calculated from the results of Feaster *et al.* (1946).

Storage temperature	k_s
45° F. (7·3° C.)	0·0025
70° F. (21° C.)	0·011
98° F. (37° C.)	0·038

TABLE 4.

The velocity coefficient (k_s) for the destruction of aneurin on storage of dehydrated pork.

(Calculated from the results of Rice *et al.* (1944).)

Storage temperature	k_s
27° C.	0·072
37° C.	0·43
49° C.	1·5
63° C.	8·9

There is a general similarity between the values for the different products in spite of pH differences, tomato juice on the whole showing the most favourable environment for aneurin retention. In only two cases, orange juice and apricots, do results for three different temperatures make it worth while to construct a curve for the Arrhenius equation. These curves are shown in Fig. 4. In spite of the necessarily approximate values for k_s and the wide scales used, there is clear confirmation of the validity of the Arrhenius equation and an indication of the value of the kinetic approach to this kind of study.

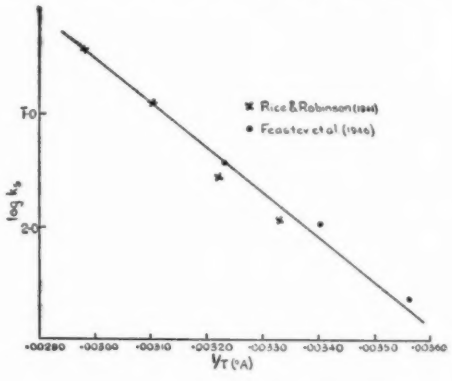

Fig. 5.

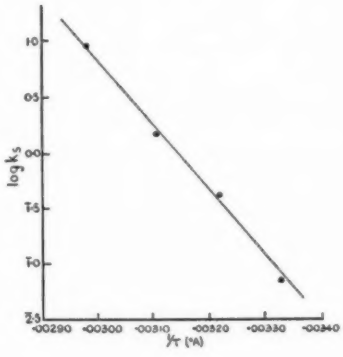

Fig. 6.

Fig. 5. Arrhenius Curve for Vitamin B_1 destruction in pork luncheon meat using k_s calculated from the results of Rice and Robinson (1944) and Feaster *et al.* (1946).

Fig. 6. Arrhenius Curve for B_1 destruction on storage of dehydrated pork using k_s calculated from the results of Rice *et al.* (1944).

There have also been recorded results for canned and dehydrated pork from which it is possible to calculate k_s quite readily and the values obtained are tabulated above (Tables 3 and 4).

In most cases, k_s could be calculated with certainty; but at the two highest temperatures, 120° and 145° F., the values must be regarded as only approximate. Nevertheless, Fig. 5, $\log k_s/1/T$ (an expression of the Arrhenius equation) for the destruction of aneurin in canned pork shows that the values of k_s obtained at these temperatures must be close to the actual

figures. It is also apparent from Fig. 5 that the results of the two groups of workers are fundamentally the same.

Rice *et al.* (1944) have studied the stabilization of aneurin in dehydrated foods and in the course of their work obtained figures for aneurin loss from dehydrated pork at six temperatures over a period of three weeks. In this time there was no detectable loss at —29° and 3° C. but values of k_s could be calculated for the four higher temperatures (see Table 4) although that for 63° C. depends on the measure of the amount remaining after only one time interval. Fig. 6 is a graph of the Arrhenius equation for aneurin loss from dehydrated pork using the values of k_s calculated from Rice *et al.* (1944).

Nymon and Gortner (1948) studied the effect of temperature, incorporation of soya flakes, container and moisture content on the retention of aneurin in dehydrated pork loaves. They recorded their results as γ/gm. on a dry, fat-free basis and from their figures (which were also graphed) they concluded:

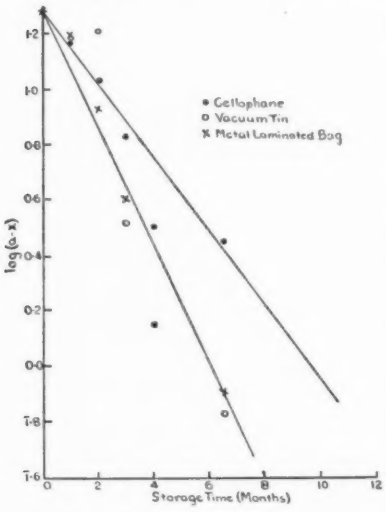

Fig. 7. log $(a - x)/t$ Curves for storage of dehydrated pork loaves in various packaging materials at 100° C. (Calculated from the figures of Nymon and Gortner, 1948.)

(i) Aneurin in the pork extended with soya flakes was more stable than that in the pork control.

(ii) In the dehydrated pork which contains soya flakes there is greater destruction of aneurin in metal and metal laminated containers than in Cellophane. (This is linked by Nymon and Gortner with the further loss of moisture through the Cellophane, but this paper is not concerned with discussion of that phenomenon.)

(iii) "There is some indication that the thiamin in the unextended, dehydrated meat may also be more stable at high temperature when packaged in Cellophane but the differences are not sufficiently large to be considered significant."

Now, it is possible to calculate k_s with fair accuracy from Nymon and Gortner's figures (e.g. Fig. 7 shows the curve log $(a - x)/t$ for the control pork loaves at 100° C.) and these values are tabulated (Table 5).

This Table gives a quantitative expression of Nymon and Gortner's conclusions. Firstly, aneurin is destroyed twice as fast in the control as in the soya-containing loaves at 70° F., at which temperature the complicating factor of appreciable drying out does not occur. Secondly, at 100° F. the rate of destruction of aneurin in the soya-containing dehydrated pork is three times as fast in the moisture proof packages as in the Cellophane. Finally, far from there being any doubt, there is no doubt at all regarding the slower rate of destruction of aneurin in the pork control when packed in Cellophane as against the metal laminated bags. This follows from the linear curves in Fig. 7, which are significantly different. The figures for the vacuum tin are plotted in this same figure. No line has been drawn but they correspond roughly with that for the metal laminated bag.

Values of k_s for Nymon and Gortner's experiments do not fit on the log $k_s/1/T$ curve (Fig. 6) for dehydrated pork (Rice *et al.*, 1944). However, both sets of authors and particu-

larly Rice and his co-workers emphasize the importance of the actual moisture content of dehydrated pork on the stability of aneurin and it is, therefore, likely that this lack of agreement is a function of different moisture contents.

TABLE 5.

Rate of destruction of aneurin (k_s) on storage of dehydrated pork loaves.

(Calculated from the results of Nymon and Gortner (1948).)

Sample	k_s at 70° F.	k_s at 100° F.
(a) Control		
Packed in Cellophane	0·048	0·079
Packed in vacuum tin	0·051	
Packed in metal laminated bags	0·051	0·13
(b) Extended		
Packed in Cellophane	0·026	0·024
Packed in vacuum tin	0·026	0·071
Packed in laminated bags	0·026	0·071

Very little information is available on the behaviour of aneurin on the storage of cereals but a recent paper by Brenner, Dunlop and Wodicka (1948) discusses the loss of aneurin when canned bread is stored at 72° and 100° F. Average percentage aneurin retentions after storage for six months and twelve months are given and from these approximate values of k_s were calculated, viz. 0·0059 at 72° F. (22° C.) and 0·023 at 100° F. (37·7° C.). These values are only approximate but, quite clearly, the kinetic interpretation of results could be used.

The results arrived at in this paper suggest that, from the point of view of aneurin retention, fruit and vegetable products fall into one group and the high protein cheese and pork into another, with, perhaps, the cereals (cf. bread) intermediate; aneurin destruction being significantly more rapid in the cheese-pork group.

From the experimental data presented and the discussion of the results obtained by other workers, it is apparent that the ordinary concepts of reaction kinetics can be applied to studies of the destruction of vitamin B_1 (and other vitamins, too, no doubt) during the storage of foodstuffs. The advantages of doing this and calculating k_s (or k multiplied by some power of 10) for each set of conditions are very obvious. It would be possible, for example, to compare far more quickly and conveniently than at present the results of similar experiments carried out in different laboratories and, even more important, it enables, through the Arrhenius equation, some prediction to be made regarding the "life" of vitamin B_1 in foodstuffs which have to be stored at any tropical or sub-tropical temperatures.

With the sole exception of canned pork where it has been shown in this paper that similar results were obtained by two different groups of investigators, it has not been possible to correlate results for foodstuffs in the same general groups.

SUMMARY.

It is proposed that the thermal destruction of vitamin B_1 during the storage of foodstuffs be followed kinetically using the equation for a first order reaction and measuring time in weeks (instead of minutes) to give a velocity coefficient, k_s, which is approximately equal to $k \times 10^4$. This constant has been used in a study of losses of vitamin B_1 from processed cheese and in the assessment of published results on certain canned foods and dehydrated pork. It is shown that the Arrhenius equation relating reaction velocity and temperature applies at storage temperatures and that the approach to storage losses through reaction kinetics can lead to a more effective interpretation of results, an easier comparison of work carried out in different laboratories and, where an Arrhenius curve has been constructed, the prediction of the vitamin B_1 retention where foodstuffs are to be stored at atmospheric temperatures.

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THE ACTION OF PROTAMINE ON BLOOD PLASMA

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Apparently W. H. Thompson (1900) was the first worker to observe that the addition of the protamine salmine to shed-blood inhibits coagulation. From a study of this phenomenon, carried out by Chargaff (1938), it appears that protamines react with phosphatides and thus inactivate the thromboplastin required for the conversion of prothrombin into thrombin.

Protamines added to plasma in concentrations higher than necessary to delay coagulation precipitate fibrinogen, as Mylon, Winternitz and de Sütö-Nagy (1942) have found. Their method is recommended for the quantitative estimation of fibrinogen.

Further, Rocha e Silva, Andrade and Teixeira (1946) reported that in dog's blood protamine accelerated the fibrinolysis induced by peptone or anaphylactic shock. Ferguson (1940) has shown that although protamines are inhibitors of the first stages of the clotting process, they have a marked accelerating effect on the thrombin-fibrin reaction.

In a previous paper from this Institute, Fantl and Simon (1948) reported that convulsions in humans, produced by intermittent current, were followed by transient fibrinolysis, and fibrinogen was not attacked.

On the other hand, Mylon *et al.* (1942) state that fibrinogen precipitated by protamine undergoes breakdown in plasma whilst in the presence of protamine and calcium ions, fibrin is formed which is quite stable. It would appear then that under certain conditions fibrinolysis can occur and under others, fibrinogenolysis. It was thought of interest to test this hypothesis. To this end, a detailed study of the behaviour of salmine towards the plasma components was carried out.

EXPERIMENTAL.

Venous blood was obtained from normal donors and collected in 1/9th of its volume of 0·1 M sodium oxalate. By differential centrifugation, cell-containing or cell-free specimens were prepared.

In the experiments to be described, the plasma specimens were divided into 5 aliquots. In the first, fibrin was estimated by recalcification. 1 ml. of plasma was measured into 25 ml. 0·85 p.c. sodium chloride and 1 ml. 0·25 M calcium chloride solution was added. These specimens were kept at room temperature for 1–2 hours, the fibrin gel which was formed was col-

[1] Working under a grant from the National Health and Medical Research Council.

lected and washed with saline until free of soluble proteins. The protein nitrogen content of this gel was determined by the biuret technique of Robinson and Hogden (1940). (Column 1, Table 1.)

In the second, the specimens were incubated without the addition of calcium ions in order to find the extent of normal fibrinogenolysis.

Specimens were kept for 21 hours at 37° C., and calcium chloride added after this time. The results are recorded in column 2, Table 1.

In a third series, the fibrin concentration was estimated by the addition of salmine sulphate and calcium ions. 1 ml. of 1 p.c. salmine sulphate dissolved in 0·85 p.c. saline was added to the diluted specimens. The mixtures were kept at + 6° C. and after 1 hour, calcium chloride was added and biuret reactions carried out on the precipitates. Results are given in column 3, Table 1.

In order to find whether there was any fibrinolysis due to salmine, fibrin was formed by the addition of calcium ions in the presence of salmine sulphate and the mixture was incubated for 21 hours at 37° C. Results are recorded in column 4, Table 1.

TABLE 1.

Influence of salmine sulphate on recovery of human plasma fibrinogen.

Fibrinogen estimations				
In absence of salmine sulphate		In presence of salmine sulphate		
1	2	3	4	5
37	—	41	42	—
38	—	44	45	38
36	33	—	—	35
33	34	—	—	33
29	—	—	29	—
32	—	—	—	34
54	52	—	—	53
41	36	—	—	37
40	37	44	39	38
* 48	53	—	—	42
44	43	—	—	45
45	47	—	—	46
41	40	—	—	40
* 47	44	—	—	46
45	41	—	—	43

Figures are given in mg. protein nitrogen per 100 ml. of plasma.

1. Ca++ added at 0 hour.
2. Ca++ added after 21 hours at 37° C.
3. Ca++ added after 1 hour at 6° C.
4. Ca++ added at 0 hour and clot incubated for 21 hours at 37° C.
5. Ca++ and thrombin added after 21 hours at 37° C.

* plasma containing cells.

Finally, the influence of salmine sulphate on fibrinogenolysis was investigated. Plasma specimens were incubated at 37° C. for 21 hours in the presence of salmine sulphate. Calcium ions and thrombin were added. Fibrin was collected and protein nitrogen estimated as previously (Column 5, Table 1).

From Table 1 the following conclusions can be drawn. Results given in columns 1 and 2 are in satisfactory agreement indicating that appreciable fibrinogenolysis did not occur in the blood samples used. A comparison of the results tabulated in columns 3 and 4 indicates that salmine did not induce fibrinolysis. With regard to the experiments recorded in column 5, it was noticed that the fibrinogen precipitated by salmine went into solution during the incubation period, and the subsequent addition of calcium ions did not produce a fibrin clot in the majority of cases. It is to be remembered, however, that in addition to calcium ions, prothrombin and thromboplastin are required for coagulation. Protamines inactivate thromboplastin and therefore no thrombin formation can take place. However, the addition of thrombin to salmine-treated plasma resulted in the recovery of fibrinogen in most cases, as indicated by the figures given in column 5.

These experiments show that the disappearance of the precipitated fibrinogen was not due to its breakdown. It seemed of interest to study the mechanism of the fibrinogen disappearance in the presence of salmine sulphate.

The most likely explanation of the phenomenon is that salmine sulphate is attacked by a plasma enzyme and the breakdown products do not share the fibrinogen-precipitating properties of the original protamine. H. C. Hagedorn (1938) detected a protamine-splitting enzyme in blood serum. He, and later H. O. Bang (1946), measured the protamine breakdown by alteration of the turbidity of a protamine-insulin suspension. We found that the extent of protaminolysis could readily be followed by the application of the Sakaguchi colour reaction (1925). In contrast to salmine sulphate, its breakdown products are soluble in trichloracetic acid.

The salmine sulphate (Lilly), used in this work, contained 22·04 p.c. total nitrogen (micro-Kjeldahl), and 58·9 p.c. arginine when estimated by the flavianate technique of Vickery (1940). The arginine hydrochloride employed contained 25·7 p.c. total nitrogen, from which a purity of 96·5 p.c. was calculated.

The colorimetric technique adopted was a slight modification of the procedure recommended by Albanese, Irby and Saur (1946). They state that either arginine, albumin or globulin can be used as standard, implying that the colour intensity of free and combined arginine is the same. This statement, however, has been contradicted for serum proteins by Keyser (1948).

The following procedure was carried out: Solutions of both 2 and 4 per cent. plasma or serum in 0·02 M phosphate buffer (pH 7·4) containing 0·59 p.c. sodium chloride were incubated with 240 mg. p.c. salmine sulphate for 4–20 hours at 37° C. The addition of an equal volume of 20 p.c. trichloracetic acid solution stopped the reaction and precipitated serum proteins and unchanged salmine sulphate. Aliquots of the filtrate were used for the tests.

Reagents required:

(a) 10 p.c. sodium hydroxide.

(b) 0·1 p.c. re-sublimed α-naphthol in 95 p.c. ethyl alcohol.

(c) 0·096N sodium hypochlorite. 12·8 gm. sodium hydroxide in 250 ml. distilled water chilled thoroughly. Chlorine, from 4 gm. potassium chlorate and 50 ml. 15 p.c. hydrochloric acid, passed in. Stock solution was kept at +6° C. and diluted to 0·096 N. as required.

(d) 20 p.c. urea solution.

Technique.

To 1 ml. of a solution containing 10 p.c. trichloracetic acid, add 1·25 ml. sodium hydroxide (a), and 3·75 ml. water. 1 ml. reagent (b) is added with vigorous shaking, and 5 minutes later, 1 ml. sodium hypochlorite (c) is added. Exactly 3 minutes later the reaction is com-

pleted by the addition of 2 ml. urea solution (d). The temperature is kept at +20° C. throughout the test. Colours were found to be stable for 2 hours. Results obtained following the incubation of human serum with salmine sulphate for 20 hours at 37° C. are recorded in the following Table:

TABLE 2.

Measurement of salmine sulphate breakdown by human serum as followed by the Sakaguchi colour reaction, using both salmine sulphate and arginine hydrochloride as standards.

Salmine sulphate	Arginine hydrochloride
0·507	1·18
0·349	0·795
0·266	0·605

Figures are expressed as mg. of arginine.

It is apparent from the figures that when salmine sulphate was used as the standard for the colorimetric estimation of arginine in the breakdown products, considerably lower readings were obtained than when arginine hydrochloride was used. In fact, while salmine sulphate gives theoretically possible values, arginine hydrochloride gives results above 100 p.c. of the theoretical. Studies of this discrepancy and its implications have been carried out and will be reported elsewhere. It was ascertained that the arginase of human serum under the above experimental conditions was inactive. Because of the unknown nature of the protamine breakdown products, it is quite apparent that colorimetric arginine estimations under our experimental conditions cannot be applied to determine the extent of protaminolysis by serum.

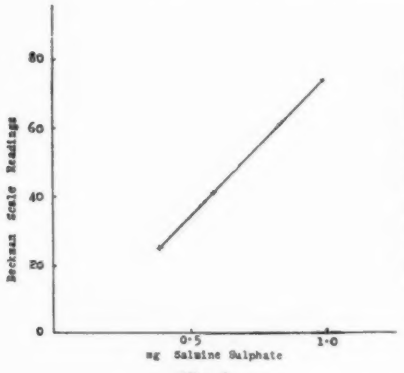

Fig. 1.

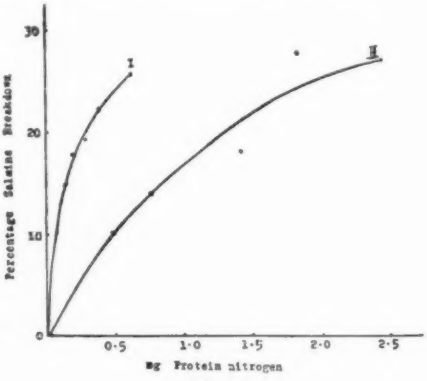

Fig. 2.

Fig. 1. Nephelometric estimation of salmine.

Fig. 2. The incubation mixtures contained 240 mg. p.c. salmine sulphate. The reaction was allowed to proceed for 6 hours at 37° C. The abscissa represents the enzyme concentration in terms of protein nitrogen.

Another method which has been tested to follow the salmine breakdown by the plasma enzyme is based on the measurement of turbidity of the heparin-protamine complex. After preliminary experiments, the following technique was found to be reproducible. To 4 ml. of a solution containing between 0·2–1 mg. salmine sulphate in 0·02 M phosphate buffer (pH 7·4) and 0·58 p.c. sodium chloride was added 1 ml. of a diluted heparin solution (Evans) corresponding to 1 mg. (100 units). It is essential to add the heparin solution quickly and under rigidly standardized conditions, preferably from a bulb pipette. A stable collodial suspension of the protamine-heparin complex is obtained, the density of which was read in the Beckman spectrophotometer using the fluorescence accessory. Fig. 1 indicates that this technique can be used in the range of 0·2–1 mg. salmine sulphate.

Results of the nephelometric and colorimetric techniques, using salmine sulphate as standard in both cases, are given in Table 3. Great discrepancies are apparent. The nephelometric technique gave appreciably lower values than the colorimetric. The results indicate that the salmine breakdown products still give precipitates with heparin.

TABLE 3.

Percentage breakdown of salmine sulphate.

Serum	Nephelometric method	Colorimetric method
1	18	64
2	16	69
3	31	90
4	20	66

Colorimetric and nephelometric methods gave only limited information of protaminolysis. It was therefore thought advisable to apply a less ambiguous method for quantitative purposes. The estimation of non-protein nitrogen by the Kjeldahl procedure was selected, using the technique of Miller and Houghton (1945).

Since experiments carried out in the range of pH 6·9–8·2 for an incubation period of four hours gave variations not greater than 10 p.c. the following experiments were all carried out at a pH of 7·4. Incubation mixtures were prepared as stated previously, and estimations carried out on 2 ml. of the trichloracetic acid filtrate.

In order to find out whether the protaminolytic enzyme is inhibited in serum, enzyme fractions were prepared by precipitation at 33 p.c. ammonium sulphate saturation and also by acetic acid precipitation at pH 5·2 from ten-fold diluted serum at 0° C.

The residue of the acid precipitation was dissolved in saline/buffer (pH 7·4) (Fig. 2, Curve I). The activity of this preparation was compared with breakdown rate of salmine sulphate by varying serum concentrations. (Fig. 2, Curve II).

The results plotted in Fig. 2 indicate that the ratios of breakdown rates in the two preparations are similar for comparable enzyme concentrations. This is interpreted to mean that antiprotaminase activity is not apparent when serum concentrations of 1–5 p.c. in the reaction mixture are used.

It appeared of some interest to obtain information with regard to the source of this blood enzyme. Total pancreatectomy and splenectomy were carried out in a dog. Success of the operation was judged 40 days later by a post-mortem examination in which no pancreatic or

splenic tissue could be found. In the post-operative period, the animal developed diabetes and ketonuria which was controlled by twice-daily injections of insulin. Twenty and forty days after the operation, protaminolytic activity of the serum was measured by using the Kjeldahl technique.

TABLE 4.

Protamine breakdown of dog sera.

Serum concentration	Control dogs		Pancreatectomized and splenectomized After 20 days		After 40 days	
	3½ hrs.	20 hrs.	3½ hrs.	20 hrs.	3½ hrs.	20 hrs.
2 p.c.	·104–·612	·544–1·488	·236	1·024	·28	1·104
4 p.c.	·216–·672	1·00–1·82	·320	1·304	·376	1·392

Time in hours indicates incubation period of the reaction mixture.
Results are given in mg. non-protein nitrogen. At the start the mixtures contained 1·96 mg. salmine N.

The physiological variations of the protaminolytic activity of dog sera were determined in blood specimens obtained from 4 dogs (2 male and 2 female).

As can be seen from Table 4 protaminolysis following the operation was within the physiological range, therefore neither pancreas nor spleen can be considered as major source of the blood enzyme.

The only other organ tested for protaminolytic activity was rabbit's liver. Following saline perfusion, *in situ*, the livers were removed and pulped through muslin. A suspension, containing 12·5 p.c. pulped liver in phosphate-saline buffer, pH 7·4 was incubated with salmine sulphate corresponding to 49 mg. p.c. protein nitrogen. After 4 hours' incubation at 37° C., 73·5 p.c. of the added salmine sulphate (corrected for the blank) was found as non-protein nitrogen. Following 21 hours' incubation, all the protein nitrogen appeared as non-protein nitrogen in the filtrate. These results indicate the presence of a very potent protaminolytic enzyme system in the liver. When similar experiments were carried out using the Sakaguchi colour reaction as indicator of the breakdown of salmine, negative results were obtained. This observation suggests that liver contains, in addition to protaminase, enzymes capable of breaking down the peptide chain to arginine which in turn is split by arginase into ornithine and urea, neither of which give a positive Sakaguchi test.

DISCUSSION.

The action of protamines on the components of the blood coagulation system is of a complex nature. The influence on prothrombin and thromboplastin is inhibitory and apparently these factors are destroyed. In contrast, the precipitation of fibrinogen by protamine is reversible, owing to the presence of a protaminolytic enzyme in plasma. Evidence for the correctness of this view can be derived from the following facts. Soluble fibrinogen obtained following incubation of plasma with salmine can be converted by thrombin into fibrin. Thrombin is a specific enzyme for this purpose and no other substrate is known which can be converted by thrombin into a water-insoluble protein. Further, the amount of salmine required for fibrinogen precipitation disappears completely during the time the precipitated fibrinogen redissolves.

The protaminolytic enzyme was found to be present in the plasma and serum of man, dog, rabbit, rat and guinea-pig. It is a well established fact that when proteolytic enzymes appear in serum, or when added to serum, they are inhibited by a normal serum component. However, no evidence for an anti-protaminase could be detected. An enzyme specific for the breakdown of basic proteins was isolated by Waldschmidt-Leitz, Ziegler, Schäffner and Weil (1931) from pancreas. The surgical removal of the pancreas as well as the spleen from a dog did not diminish the protaminase activity of the serum. On the other hand, rabbit's liver was found to be a potent source of protaminolytic activity. This effect, however, could be shown only by the increase of non-protein nitrogen during incubation. The Sakaguchi colour reaction gave negative results in the liver filtrates, indicating that, in addition to protaminase, enzymes capable of splitting salmine breakdown products are also active.

SUMMARY.

The precipitation of fibrinogen from human plasma by salmine sulphate is a reversible process. Owing to the presence of a protaminase, salmine is broken down into products soluble in trichloracetic acid which do not share the fibrinogen precipitating properties of salmine. Blood protaminase is present in a number of animals. In contrast to other proteolytic enzymes in serum, protaminase is not counteracted by inhibitors. Pancreatectomy and splenectomy have no influence on the enzyme concentration in blood plasma. *In vitro* experiments point to the liver as a potent source of protaminolytic activity.

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STUDIES OF THE FLORA OF THE MOUTH

I. YEAST-LIKE ORGANISMS: SOME MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS

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Since Miller (1890) expressed the ideas now commonly included in the well-known chemico-parasitic theory of dental caries and since Goadby (1903) catalogued the micro-organisms of the human mouth, various species of bacteria individually have been suggested as the principal or sole parasitic agent in the causation of that disease. Although lactobacilli have attracted most attention, some workers have attributed a more important rôle to the streptococci (Bibby, 1940; Anderson and Rettger, 1937; Clarke, 1924; Tunnicliff and Hammond, 1938) or to the combined action of several species of micro-organisms (Hansen, 1937; Fosdick, 1937; Snyder, 1940).

The symbiotic and synergistic relationships between different species of micro-organisms appeared to us to be worthy of study, because it is clear that bacteria operating in the oral cavity, except possibly in protected sites, are compelled to do so in the presence of numerous other organisms and enzymes. Fosdick (1937) has discussed a possible mechanism of symbiosis between lactobacilli and yeast.

Colonies of yeast-like cells have been observed frequently on tomato-peptone agar medium when inoculated with saliva. However, Knighton (1939) found that the majority of so-called yeasts isolated from saliva belonged to the genus Candida (frequently described as Monilia). We therefore decided, as a preliminary to an investigation of synergistic actions of bacteria isolated from the mouth, to study the yeast-like organisms (hereinafter, for convenience, termed "yeasts") and this report is concerned with our observations on some of their morphological and physiological characteristics, together with a classification of the strains examined.

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1 Part of this work was carried out while holding an Australian Dental Association, N.S.W. Branch Scholarship, and the Walter and Eliza Hall Dental Research Scholarship of the University of Sydney.

2 Part of this work was carried out with the aid of a grant from the National Health and Medical Research Council of Australia.

MATERIALS AND METHODS.

Strains were isolated from (1) University students and persons attending the United Dental Hospital of Sydney, (2) boys at Boys' Town, Engadine, New South Wales and (3) natives in New Guinea.

Activated saliva was obtained by instructing the subject to chew a piece of paraffin for five minutes and to collect the saliva in a 6 inch x 1 inch test tube. Table 1 summarizes the methods used to study these samples of saliva and the micro-organisms isolated from them.

TABLE 1.

Outline of methods used in this study of yeast-like organisms.

	Group		
	1.	2.	3.
	University students and persons attending the United Dental Hospital of Sydney	Boys at Boys' Town, Engadine, N.S.W.	Natives in New Guinea
Saliva	0·1 ml. of (a) undiluted and (b) ten-fold diluted, spread over separate tomato-peptone agar plates	3 drops of undiluted (delivered from a pasteur pipette) spread over tomato-peptone agar plates	Same as for Group 1
Incubation of plates	37° C. for 3 or 4 days.		
	Subcultures made from selected colonies and stored for future study on:		
	Robertson's cooked meat medium and later glucose agar slopes	Glucose agar slopes	Same as for Group 1
	Strains recovered from stock cultures, purified when necessary, and subcultured on glucose agar.		
Strains examined for:			
Growth	Tomato-peptone agar 1 p.c. glucose agar 10 p.c. sheep blood agar 1 p.c. glucose broth	By the methods described by Martin *et al.*, 1937.	
Fermentation	1 p.c. substrate in peptone water (Readings made at intervals over a 14-day period.) Voges-Proskauer test Methyl red test		
Ascospore-production	Vegetable extract agar (Mrak *et al.*, 1940)		
Mycelium-chlamydospore-formation	Cornmeal extract (Anderson, 1946)		
Proteolysis	Loffler's coagulated serum medium Gelatin stab		
Classification based on	Cultural, morphological and physiological features	By the method used by Martin *et al.*, 1937.	

RESULTS.

One hundred and sixty-six strains were isolated from the different groups of subjects. For convenience the results are recorded separately for each of the three groups.

1. *Sixty-eight strains obtained from students and patients.* (a) On tomato-peptone agar. After 72–96 hours' incubation the colonies were approximately 3–6 mm. in diameter, quite opaque with a matt surface, obtusely conical in shape, whitish in colour and butyrous in consistency.

(b) On 1 p.c. glucose agar. After 24 hours' incubation the colonies were approximately 1 mm. in diameter with a somewhat shiny surface and an entire edge. After 72 hours they had grown considerably larger and in general resembled those on tomato-peptone agar.

(c) On blood agar. After 10 days' incubation the majority of strains formed grey-white, matt colonies, 4–6 mm. in diameter. Two strains formed smaller (2–3 mm. diameter), white, matt colonies, whereas five strains showed white, shiny colonies. A mycelial fringe was observed around colonies of another strain.

(d) In 1 p.c. glucose broth. After 72 hours' incubation the cultures showed a heavy deposit and a clear supernatant fluid. Most cultures showed some fragments of growth attached to the tube at the top of the fluid; occasionally a powdery pellicle was visible.

Films prepared from the 72-hour glucose-broth cultures were stained by Gram's method and examined microscopically. The films showed large Gram-positive budding ovoid cells. A minority of cells were elongated to several times the average length.

Biochemical Characteristics.

Fermentation. The results of the fermentation tests are set out in Tables 2, 6 and 7. In Table 2 these results are correlated with other properties. No culture reacted to the methyl red and Voges-Proskauer tests.

Proteolysis. No strain showed any proteolytic properties when grown on Loeffer's coagulated serum medium or in gelatin stab cultures.

The sixty-eight strains studied were classified according to cultural, morphological and physiological features (Table 2).

TABLE 2.

Classification of sixty-eight strains of "yeasts" from students and patients.

No. strains	P.c. strains	Growth on			Fermentation				Classification
		1 p.c. glucose broth (48 hr.)	blood agar	cornmeal extract	G	S	L	M	
56	82·3	deposit, clear supernatant	grey-white matt, colony 4–6 mm. diam.	mycelium chlamydo-spores	AG	A	—	AG	*Candida albicans*
2	2·9	surface film	small, white colony	budding cells	AG	—	—	—	*C. krusei*
1	1·5	surface growth	grey-white colony with mycelial fringe	pseudo-mycelium	AG	AG	—	AG	*C. tropicalis*
2	2·9	deposit	white, shiny colony	budding cells	AG	AG	—	A	Yeast
3	4·4	deposit	white, shiny colony	pseudo-mycelium	AG	A	—	A	Yeast
2	2·9	deposit	white, matt colony	pseudo-mycelium or budding cells	AG	A	—	AG	*? C. albicans*
2	2·9	deposit	small, grey-white colony	budding cells	AG	AG	—	AG	*? C. tropicalis*

G = glucose, S = sucrose, L = lactose, M = maltose.
AG = acid and gas produced, A = acid produced, — = no acid or gas produced.

2. *The fifty-seven strains obtained from boys at Boys' Town (Group 2) and the forty-one strains from New Guinea natives (Group 3)* were examined by the methods of Martin, Jones, Yao and Lee (1937). The results obtained are shown in Tables 3 and 4 respectively.

TABLE 3.

Classification of fifty-seven strains of "yeasts" from boys at Boys' Town.

No. strains	P.c. strains	Growth on				Ascospores	Fermentation				Classification
		Sabouraud's glucose broth (48 hr.)	Blood agar	Cornmeal agar	Cornmeal extract		G	S	L	M	
34	59·7	deposit, no surface growth	grey, smooth colony 4–6 mm. diam.	branching mycelium, terminal and lateral chlamydospores	mycelium chlamydospores	none	AG	A	—	AG	*C. albicans*
6	10·5	surface growth	grey, white irregular colonies 1–4 mm. diam.	mycelium and elongated blastospore	as on cornmeal agar	none	AG	—	—	—	*C. krusei*
2	3·5	no surface growth	small colonies	mycelium ill-developed	elongated blastospores	none	AG*	—	—	—	*C. parakrusei*
5	17·5 (10)	no surface growth	grey-white irregular small colonies	no mycelium	large budding cells	2–4 oval	AG	AG	—	AG	Yeast
1		no surface growth	grey white irregular small colonies	no mycelium	small budding cell	2–4 oval	A	A	—	A	
3		no surface growth	creamy high-domed colonies 1–2 mm. diam.	no mycelium	budding cells	2–4 oval	AG	AG	—	A	
1		no surface growth	grey-white irregular colonies	no mycelium	budding cells	2–4 oval	AG	AG	AG	—	
1	5·2 (3)	deposit	rough-grey colonies 3 mm. diam.	no mycelium	budding cells	none	AG	A	—	AG	? *C. albicans*
1		deposit	grey, smooth colonies, 4–6 mm. diam.	mycelium; terminal and lateral chlamydospores	mycelium large intercalary chlamydospores	none	A	A	—	AG	
1		deposit	grey, smooth colonies 4–6 mm. diam.	no mycelium	mycelium; no chlamydospores	none	AG	A	—	AG	
1	1·7	deposit	grey-smooth colonies 4–6 mm. Mycelial fringe	mycelium ill-developed	mycelium and intercalary chlamydospores	none	AG	—	—	AG	? *C. stellatoides*
1	1·7	deposit	grey-smooth colonies 4–6 mm. diam.	mycelium; elongated blastospores	? Growth	none	A	AG	—	—	?

* One strain produced only acid in glucose.

TABLE 4.

Classification of forty-one strains of "yeasts" from natives of New Guinea.

No. strains		P.c. strains	Growth on 1 p.c. glucose broth	Blood agar	Cornmeal agar	Fermentation G	S	L	M	Classification
28		68·3	deposit; clear supernatant	round, smooth obtusely conical colonies, 4–6 mm. diam.	mycelium chlamydospores	AG	A	—	AG	*C. albicans*
3*			deposit; surface film and/or collar	round, smooth, grey, shiny colonies, 2–4 mm. diam.	no mycelium; budding cells	A	A	—	—	
5			deposit; surface film and/or collar	round, smooth, grey, shiny colonies, 2–4 mm. diam.	no mycelium; budding cells	AG	AG	—	a	
1	12	29·3	deposit; surface film and/or collar	round, smooth, grey, shiny colonies, 2–4 mm. diam.	no mycelium; budding cells	AG	A	—	—	Yeast
2			deposit; surface film and/or collar	round, smooth, grey, shiny colonies, 2–4 mm. diam.	no mycelium; budding cells	A	A	—	a	
1			deposit; surface film and/or collar	round, smooth, grey, shiny colonies, 2–4 mm. diam.	no mycelium; budding cells	AG	AG	—	—	
1		2·4	deposit	round colony with white aerial hyphae	mycelium; elongated blastospores	A	A	—	A	?

* On blood agar one strain gave pink colonies, 2–3 mm. in diameter, and another strain gave very small, dome-shaped, creamy colonies.

a = weak acid reaction.

TABLE 5.

Comparison of the three groups of strains of "yeasts."

Species	Total (Groups 1, 2, 3) No. strains	P.c. strains	Group 1 Students and Patients No. strains	P.c. strains	Group 2 Boys at Boys' Town No. strains	P.c. strains	Group 3 Natives in New Guinea No. strains	P.c. strains
Candida albicans	118	71·1	56	82·3	24	59·7	28	68·3
C. krusei	8	4·8	2	2·9	6	10·5	—	—
C. parakrusei	2	1·2	—	—	2	3·5	—	—
C. tropicalis	1	0·6	1	1·5	—	—	—	—
Yeast*	27	16·3	5	7·3	10	17·5	12	29·3
? *C. albicans*	5	3·0	2	2·9	3	5·2	—	—
? *C. tropicalis*	2	1·2	2	2·9	—	—	—	—
? *C. stellatoidea*	1	0·6	—	—	1	1·7	—	—
?	2	1·2	—	—	1	1·7	1	2·4
Total	166	100·0	68	99·8	57	99·8	41	100·0

* All strains in this group failed to produce mycelium. Some strains produced ascospores (10 from Boys' Town), the others did not.

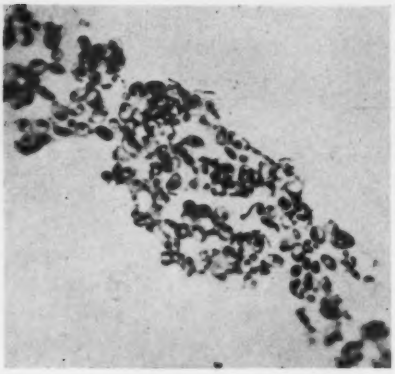

Fig. 1.

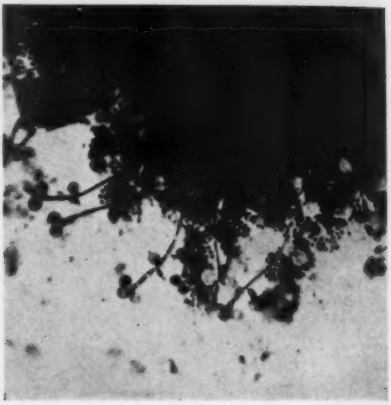

Fig. 2.

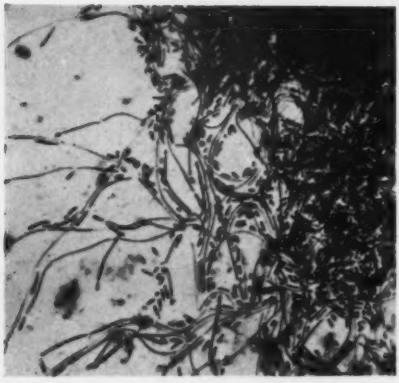

Fig. 3.

EXPLANATION OF PLATES.

Fig. 1. Ascospores in a culture of yeast incubated at room temperature for four weeks on carrot disc.

Fig. 2. Mycelium, blastospores and chlamydospores in a slide culture of *C. albicans* incubated at room temperature for several days on cornmeal agar.

Fig. 3. Mycelium and elongated blastospores in a slide culture of *C. krusei* incubated at room temperature for several days on cornmeal agar.

Figs. 1–3 illustrate various morphological features observed in the 166 strains. A comparison of the proportion of various "yeasts" in the three groups (Table 5) shows that *C. albicans* preponderates in all and that there is considerable variation between the three groups of strains in the relative proportions of *C. albicans* and yeast (see footnote, Table 5). The group from the natives showed the highest proportion of yeast (29·3 p.c.).

TABLE 6.

The fermentation of various substrates by oral strains of "yeasts."

No. strains	Glucose	Sucrose	Maltose	Galactose	Mannose	Laevulose	Trehalose
62	AG	A or AG	AG	A or AG	AG	AG	AG
3	AG	A	A	A	AG	AG	—
1	AG	A	AG	A	AG	AG	—
1	AG	—	—	—	AG	AG	AG
1	AG	—	—	—	AG	AG	—

TABLE 7.

Number of strains of oral "yeasts" which fermented various substrates.

Substrate	Acid and Gas (AG)	Acid (A)	No Acid or Gas
Glucose	68	—	—
Sucrose	5	61	2
Maltose	60	6	2
Galactose	25	41	2
Mannose	68	—	—
Laevulose	68	—	—
Trehalose*	60	3	5
Raffinose	1	—	67
Dextrin	—	1	67
Xylose	—	1	67

The following substrates were not fermented: lactose, arabinose, rhamnose, inulin, glycogen, salicin, starch, erythritol, sorbitol, adonitol, inositol, duicitol and mannitol.

* The value of this substrate for taxonomical purposes has been studied by Bouthilet *et al.* (1949). Their results and ours seem to indicate that it has little if any value for the recognition of Candida.

DISCUSSION.

The presence of "yeasts" in the mouth has been known for very many years (Miller, 1890; Goadby, 1903; Hansen and Epple, 1937; Rosebury, 1939; Knighton, 1939). Only recently, however, has their possible importance in the breakdown of carbohydrates to acids been emphasized in relation to dental caries. Pickerill's (1912) observations upon the biological activities of yeast are of interest in this connection. Nor is it surprising that "yeasts" should be accepted as part of the flora of the normal mouth, since they are very common components of the flora of the atmosphere. That many micro-organisms occur in the mouth as transients is understandable. In view of the free entry into the mouth of likely sources of "yeasts" viz. food, water, air (including dust), it might therefore be reasonable to anticipate that a search for "yeasts" in the mouth would

reveal quite a large variety of species. Our observation that the majority of "yeasts" are Candida confirms that made by Knighton in 1939. The 166 strains (selected at random) studied by us included 118 (71·1 p.c.) strains of *Candida albicans,* 8 strains of *C. krusei* (4·8 p.c.) and 27 (16·3 p.c.) strains of yeast. These percentages are similar to those calculated from the data given by Knighton (1939). However, the similarity is coincidental because each of the 105 strains studied by him was not isolated from a different individual, as were the strains studied by us.

Occasionally we found that both *C. albicans* and *C. krusei* or *C. albicans* and yeast could be isolated from the same individual. When the same species was found in one individual's saliva on several occasions, it was recorded as one strain and not several.

Two other points may be mentioned of interest to workers engaged in making lactobacillus counts (Kulp and White, 1932) as a test for caries-susceptibility. First: the tomato-peptone agar medium used for this purpose supports the growth of both Candida and yeasts quite well, and it is often impossible to differentiate their colonies by macroscopic inspection; both may be large, conical, white or creamy with matt surface. Second: occasionally there were seen distinctive colonies, 6–9 mm. in diameter, flat and somewhat corrugated, grey in colour, with matt surface and undulating edge. These proved to be *C. krusei.*

Another noteworthy feature is the size of the colonies of *C. albicans* on sheep blood agar. Knighton records a larger size than that observed by Martin, Jones, Yao and Lee. We observed large colonies, as Knighton did.

SUMMARY.

The morphological and physiological characteristics are described for 166 strains of yeast-like organisms isolated from the mouth. These strains were studied in three groups according to their origin, viz. two groups from white Australians and one group from New Guinea natives. The strains were classified and a comparison was made of the proportion of each species within each group. Variation in the proportion of each species in the groups was noted. Approximately 71·1 p.c. of all the strains were classed as *Candida albicans,* 4·8 p.c. as *Candida krusei* and 16·3 p.c. as yeasts.

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STUDIES OF THE FLORA OF THE MOUTH

II. YEAST-LIKE ORGANISMS: SEROLOGICAL PROPERTIES

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The interpretation of specific agglutination reactions of yeast-like organisms is often difficult, due (at least in part) to the large size of the organisms and the consequent rapidity of sedimentation of the cells. Benham (1931) tried to overcome these difficulties by using 0·25 ml. of a saline suspension of antigen and 0·25 ml. of diluted antiserum and shaking the mixture for half an hour in a Kahn shaking apparatus at room temperature. The degree of agglutination was recorded and the tubes were placed in the refrigerator overnight. They were then shaken again for half an hour before the final reading was made. Benham reported that this procedure produced higher agglutination titres than incubation in a water bath at 56° C. Other investigators have used slide-agglutination techniques with various degrees of success.

Norris and Rawson (1947), using the ordinary tube method of performing agglutination tests, allowed the cells to settle and then after gently tapping the tubes observed the size of the clumps by means of a concave mirror. They graded the clumps according to size and shape and checked the agglutination by microscope.

The observation and assessment of agglutination of red blood corpuscles are well-known to be influenced by rapid sedimentations of the cells, and Landsteiner and Wiener (1941) have made use of sedimentation-patterns to detect the presence of Rh agglutinogen in human blood-corpuscles. It occurred to us that the techniques used for performing haemagglutination tests might be applicable to "yeasts". Since G. K. Hirst's (1941) observation that the red blood cells of the chicken are agglutinated by the influenza virus, the test has been extensively used in studying the viruses and several variations of the original method have been described. Of these, that devised by Burnet (1942) is both simple and precise. Preliminary tests convinced us that Burnet's method

[1] Part of this work was carried out while holding an Australian Dental Association, N.S.W. Branch Scholarship, and the Walter and Eliza Hall Dental Research Scholarship of the University of Sydney.

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of observing the patterns produced by the agglutinated red cells, after they had settled into the bottom of the test-tubes, was applicable to specific agglutination reactions with "yeast" cells.

This report, then, describes (i) a method for observing the occurrence of specific agglutination of yeast-like organisms (hereinafter called "yeasts") and (ii) the results obtained by the use of this technique.

Materials and Methods.

Early experiments indicated that the addition of a dye such as neutral red rendered the clumped "yeast" cells more easily visible. Eventually malachite green was selected because in addition it seemed to render the suspension more uniform and stable, so that in the absence of agglutination the cells formed a smooth "button" of deposit. The strains of "yeast" used for this investigation were obtained from two widely different sources: 54 from university students in Sydney and 39 from natives of Pātep II in New Guinea.

Preparation of the Suspension (Antigen) for the Agglutination Tests.

In an attempt to determine the most satisfactory conditions for preparing the suspensions many experiments were conducted to determine (a) the concentration of antigen and electrolyte (NaCl) and (b) the type and concentration of dye[3] which were best suited to our purpose. In addition, observations were made to ascertain (c) the number of times the cells could be washed without affecting the stability of the suspension of antigen and (d) whether it was preferable to wash the cells by centrifugation or by allowing them to settle.

The following method was found to be satisfactory and was used throughout this study. 1 p.c. glucose agar slopes seeded with the organisms were incubated at 37° C. for 24 hours. The cells were washed off the medium with 5 ml. 0·008 p.c. malachite green in 0·1 p.c. NaCl and the suspension was kept at 37° C. for 2 hours. The suspension was then centrifugated at approximately 1,200 r.p.m. until the supernatant fluid was clear. The fluid was removed and the deposit washed once with 5 ml. 0·1 p.c. NaCl and again centrifugated. Finally the deposit was resuspended in 10 ml. 0·1 p.c. NaCl and the suspension used as the antigen.

Preparation of Antiserum.

Two groups of normal rabbits were used. A sample of blood was taken before the animals were given injections of antigen. The normal serum thus obtained was tested for the presence of naturally-occurring agglutinins.

The first group of rabbits was given interperitoneal injections of a saline (0·85 p.c.) suspension of the selected strains (No. 77, 501 and 502) equivalent in opacity to a suspension

[3] Our earliest observations were made with unstained cells but, owing to the grey-white colour of the agglutinated or sedimented masses, it was difficult to distinguish the different grades of agglutination. To ascertain whether other dyes than neutral red or malachite green might be even more effective we tested eight others by observing their ability to give a smooth button of deposit in the control tubes. Concentrations of 1 p.c., 0·2 p.c., 0·1 p.c. and 0·05 p.c. were used in 0·1 p.c. NaCl (this concentration of NaCl proved to be the most satisfactory).

The dyes were brilliant cresyl blue, methylene blue, safranin, neutral red, toluidine blue, crystal violet, methyl violet and janus green. Malachite green 0·008 p.c. was also used for comparison. Only brilliant cresyl blue (0·2 p.c., 0·1 p.c. and 0·05 p.c.), methyl violet (0·05 p.c.), janus green (0·05 p.c.) and malachite green (0·008 p.c.) were found to be satisfactory, and some strains showed autoagglutination irrespective of the dye used.

containing 2,000 M *Bacterium coli* per ml. Altogether 9 ml. (1 × 1 ml. + 4 × 2 ml.) were injected into each rabbit over a period of seventeen days. Ten days after the last injection the animals were bled by cardiac puncture and the serum separated from the clot after standing 24 hours. These antisera were used for the preliminary experiments.

The second group of rabbits was given a triple series of intravenous injections of saline (0·85 p.c.) suspensions (1 volume of packed cells in 200 of saline): one, two and three ml. were injected on the first, second and third day respectively of each of three consecutive weeks. Five strains of "yeasts" were used (*C. albicans* Nos. 2, 35 and 545; *C. krusei* No. 22, yeast No. 555) and each strain was injected into two separate rabbits. The rabbits were bled by cardiac puncture one week after the last injection. These antisera were also used in preliminary experiments. Three months later a single series of injections was given to the same animals, which were again bled by the same method as above. The antisera so obtained were used in the agglutination tests to be described below.

Slide Agglutination.

Tests were carried out by "rock-rotating" on a slide one drop of antigen and one drop of diluted antiserum for two minutes after mixing with a thin wooden rod. The antiserum was used diluted serially with 0·1 p.c. NaCl so as to obtain dilutions of 1 in 20, 1 in 40, 1 in 80, 1 in 160, 1 in 320 and 1 in 640.

Sedimentation-Agglutination.

Equal volumes (0·5 ml.) of antigen and of antiserum diluted in 0·1 p.c. NaCl were mixed by shaking in 3½ inch x ½ inch tubes. The tubes were set aside to allow the cells to settle undisturbed at room temperature. The patterns formed by the agglutinated "yeast" cells on the bowl of the tubes were observed after 2½–3 hours. A control tube containing 0·5 ml. 0·1 p.c. NaCl instead of diluted antiserum was included in each test. The patterns formed in the tubes were similar to those described by Burnet (1942) and Burnet *et al.* (1942) as occurring when suspensions of hen red blood corpuscles are treated with influenza virus (the Hirst phenomenon) and allowed to settle (see Fig. 1).

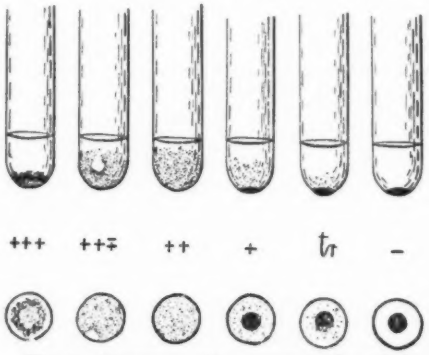

Fig. 1. Diagrammatic representation of the patterns corresponding to the grades of agglutination.

With satisfactory suspensions of antigen the titre was taken as that (final) dilution of antiserum which gave a + grade of agglutination in the sedimentation test. Some strains were encountered which agglutinated in the presence of 0·1 p.c. NaCl. It was very difficult, at times impossible, to determine the titre of sera against these strains, because the control tube showed ++ grade (or greater) of agglutination. In such instances the titre of an antiserum was taken as the highest dilution of the antiserum which caused agglutination of a grade exceeding that in the control tube by a degree represented by +. All dilutions of antisera were recorded as the final dilution in the mixtures.

RESULTS.

Preliminary Tests.

The normal rabbit sera failed to produce more than a trace of agglutination by the slide method and failed entirely by the sedimentation method. These results appear to differ from those of Drake (1943).

Slide-agglutination tests using antisera produced by intraperitoneal injection and 68 strains of "yeast" isolated from the mouth failed to reveal any noteworthy differences between strains. However, only dilutions of 1 in 2 and 1 in 20 (final concentration in the tests) were used.

Sedimentation-agglutination tests with one of these antisera gave the following results. Thirty-five strains were tested and of these 23 yielded satisfactory results in that the suspension showed no auto-agglutination whereas the remaining twelve strains showed various degrees of auto-agglutination in the control tube. The titres of the 23 satisfactory strains are shown in Fig. 2a.

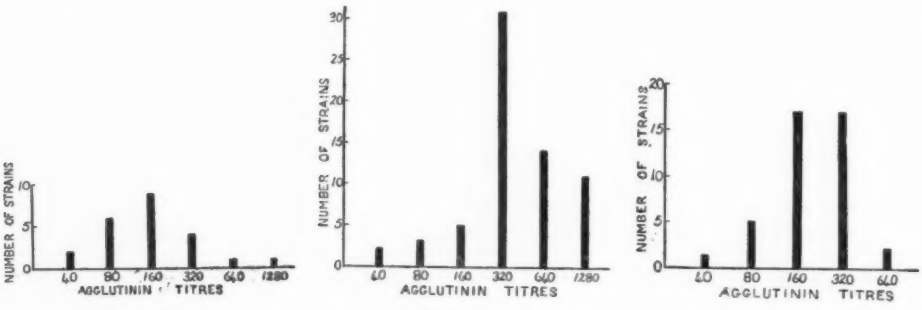

Fig. 2 (a). **Fig. 2 (b).** **Fig. 2 (c).**

Fig. 2. Distribution of agglutinin titres. (a) Preliminary tests with "yeasts." (b) Tests of *C. albicans* suspensions against homologous antibody (strain 545). (c) Tests of *C. albicans* suspensions against homologous antibody (strain 35).

Principal Series of Tests.

Tests with Strains of Candida albicans *from Sydney and New Guinea.*

i. *Against antiserum 461B,* prepared against a Sydney strain of *C. albicans* (Fig. 2b). 38 Sydney strains and 28 New Guinea strains were tested by the sedimentation technique.

56/66 (84·84 p.c.) strains were agglutinated by serum diluted 1/320 or higher,
61/66 (92·42 p.c.) strains were agglutinated by serum diluted 1/160 or higher,
64/66 (96·97 p.c.) strains were agglutinated by serum diluted 1/80 or higher,
66/66 (100·00 p.c.) strains were agglutinated by serum diluted 1/40 or higher.

In general the New Guinea strains were agglutinated at a rather higher serum titre than were the Sydney strains.

ii. *Against antiserum 458B,* prepared against a different Sydney strain of *C. albicans* (Fig. 2c).

19/42 (45·25 p.c.) strains were agglutinated by serum diluted 1/320 or higher,
36/42 (85·71 p.c.) strains were agglutinated by serum diluted 1/160 or higher,
41/42 (97·52 p.c.) strains were agglutinated by serum diluted 1/80 or higher,
42/42 (100·00 p.c.) strains were agglutinated by serum diluted 1/40 or higher.

In general the agglutination titres showed a similar distribution for both New Guinea and Sydney strains.

When the results of tests with the two different antisera are compared, it is clear that 100 p.c. of Candida strains were agglutinated by serum diluted 1/40 or higher and that 97 p.c. were agglutinated by serum diluted 1/80 or higher.

Tests with Strains of Candida albicans *from Sydney.*

i. *Against antiserum 459B,* prepared against a Sydney strain of *C. krusei.*

7/14 (50·00 p.c.) strains were agglutinated by serum diluted 1/320 or higher,
14/14 (100·00 p.c.) strains were agglutinated by serum diluted 1/160 or higher.

ii. *Against antiserum 460B,* prepared against the same strain of *C. krusei.*

7/14 (50·00 p.c.) strains were agglutinated by serum diluted 1/320 or higher,
14/14 (100·00 p.c.) strains were agglutinated by serum diluted 1/160 or higher.

Tests with Strains of Candida albicans *from Sydney and New Guinea.*

i. *Against antiserum 464B,* prepared against a Sydney strain of yeast. 38 Sydney strains and 26 New Guinea strains (64 in all) were tested and the whole sixty-four failed to show agglutination in the presence of serum diluted 1/40.

Tests with Strains of Yeast from Sydney and New Guinea.

Only 1/3 Sydney strains and only 3/11 New Guinea strains could be tested because the remainder were autoagglutinable. The Sydney strain agglutinated with serum (464B anti-yeast) diluted 1/640 and the three New Guinea strains failed to show agglutination.

Another attempt to perform these tests was entirely unsuccessful because on this occasion all the strains of these yeasts yielded autoagglutinable suspensions.

Tests with Strains of Yeast from New Guinea.

i. *Against antiserum 461B,* prepared against a Sydney strain of *C. albicans.*

2/3 (66·67 p.c.) strains were agglutinated by serum diluted 1/320 or higher,
3/3 (100·00 p.c.) strains were agglutinated by serum diluted 1/40 or higher.

ii. *Against antiserum 458B,* prepared against a different strain of *C. albicans.*

1/4 (25·00 p.c.) strains were agglutinated by serum diluted 1/320 or higher,
2/4 (50·00 p.c.) strains were agglutinated by serum diluted 1/160 or higher,
3/4 (75·00 p.c.) strains were agglutinated by serum diluted 1/40 or higher,
1/4 (25·00 p.c.) strains failed to show agglutination at 1/40.

The yeasts were not tested against an anti-*C. krusei* serum.

Tests with Strains of Candida krusei.

These could not be carried out with Sydney strains because all the suspensions were auto-agglutinable and no strains were isolated in New Guinea.

Comparison of the Slide and Sedimentation Methods.

i. *Sydney strains.* (a) 24 strains of "yeasts" were tested against antiserum 464B (anti-yeast). The homologous strain alone reacted and was agglutinated by serum diluted 1/320 on the slide and 1/640 by the sedimentation method.

(b) The same 24 strains of "yeast" together with another four strains were tested against antiserum 458B (anti-*C. albicans*). The titres obtained were approximately the same by the two methods.

ii. *New Guinea strains.* (a) 7 strains of *C. albicans* were tested against antiserum 458B (anti-*C. albicans*). The titres obtained were approximately the same by the two methods.

(b) 7 strains of *C. albicans* were tested against antiserum 461B (anti-*C. albicans*: different strain from that used to produce antiserum 458B). The sedimentation method gave results at least as good as and often better than those obtained by the slide method. A series of comparisons is shown in Table 1.

TABLE 1.

Comparison of slide and sedimentation-agglutination methods.

No. strain	Antiserum 461B		Antiserum 458B	
	Slide	Sedimentation	Slide	Sedimentation
$94A_1$	320	1280	160	640
144	320	1280	160	160
159	320	1280	320	640
224	640	1280	320	80
227a	640	640–1280	320	160–320
231	160	1280	320	160
266	320	1280	320	320

SUMMARY.

A technique is described for the observation of agglutination of Candida and yeasts. The method is an adaptation of Burnet's version of Hirst's viral haemagglutination test for influenza virus and might be applicable to the study of agglutination of any particulate matter of dimensions comparable to those of erythrocytes or "yeast" cells.

100 p.c. of strains from Sydney and New Guinea tested against two Sydney *C. albicans* antisera were agglutinated by serum diluted 1/40 or higher and 97·5 p.c. by serum diluted 1/80 or higher.

All the Sydney *C. albicans* strains were agglutinated at a titre of 1/160 or higher with two antisera prepared against one Sydney strain of *C. krusei*.

100 p.c. of 64 strains of *C. albicans* from Sydney and New Guinea tested against one anti-yeast (Sydney strain) serum failed to show agglutination by serum diluted 1/40.

Strains of *C. albicans* from Sydney and New Guinea respectively appear to be similar when examined (a) morphologically, (b) physiologically and (c) serologically.

The very meagre data available suggest that (a) yeasts are less satisfactory *in vivo* antigens than are *C. albicans* and *C. krusei* and (b) there is some cross reaction between yeast suspensions and *C. albicans* serum. Norris and Rawson (1947) obtained evidence of a similar community of antigenic components.

Of 2 strains of *C. krusei* two, of 14 strains of yeast eleven, but of 78 strains of *C. albicans* only 12, yielded autoagglutinated suspensions.

Parallel observations made by the slide and sedimentation methods show that the sedimentation method is preferable for mass testing of strains in that the time and physical effort required are less.

The evidence adduced, admittedly meagre, suggests that ordinary serological methods do not show clear-cut differentiation between *C. albicans* and *C. krusei* but that some differentiation is possible between yeasts on the one hand and Candida species on the other hand.

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STUDIES OF THE FLORA OF THE MOUTH

III. YEAST-LIKE ORGANISMS: SOME OBSERVATIONS ON THEIR INCIDENCE IN THE MOUTH

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Yeast-like organisms have frequently been observed in cultures made from saliva. In 1925 Bunting and Palmerlee noted the occurrence of colonies of "yeasts"[2] in cultures of saliva. Hatton and Fosdick (1937) reported significant differences in the incidence of "yeasts" and lactobacilli in groups of caries-susceptible and caries-immune individuals. Prader (1947) also found that the greater the incidence of caries the greater was the incidence of "yeasts"; however, the numbers in the respective groups observed were too small to permit of the establishment of a statistically significant association.

The first attempt to classify the "yeasts" of the mouth was made by Knighton (1939), who found that a large proportion of strains isolated by him belonged to the genus Candida. Fisher (1936) showed that *Candida albicans* is an inhabitant of the throats of children and adults and Todd (1937) found them in the mouths and throats of 14 p.c. of 1,000 individuals. Keiper and Prescott (1938) on the other hand could find only three students among 100 who harboured this organism in these sites.

This investigation was undertaken to determine (1) the incidence of "yeasts" in various parts of the oral cavity and (2) some of the conditions which might affect the incidence of *Candida albicans* in the saliva of different groups of individuals.

Materials and Methods.

The groups of persons examined for the presence of "yeasts" and/or *C. albicans* were selected according to age, sex, presence or absence of teeth and presence or absence of dental caries. They included (a) children (Bowral) free from dental caries, (b) children (kindergarten) with a high incidence of dental caries, (c) three groups of students with high incidence of dental caries, (d) natives in New Guinea with a low incidence of dental caries (the strains from this group were isolated by members of the Commonwealth Nutrition Survey

[1] Part of this work was carried out while holding an Australian Dental Association, N.S.W. Branch Scholarship, and the Walter and Eliza Hall Dental Research Scholarship of the University of Sydney.

[2] The term "yeasts" is used in an inclusive sense to indicate yeast-like organisms and therefore covers Candida, the true yeasts and Cryptococcus.

Party—Sinclair *et al.*, 1949), and (e) boys (an institution) who had a high incidence of dental caries.

Material for bacteriological examination was collected in one of the following ways: (a) the subject chewed paraffin for five minutes and the activated saliva was ejected into a sterile 6 inch x 1 inch test tube. (b) sterile swabs were rubbed over the teeth and gingivae. (c) carious teeth from the Exodontia Department of the United Dental Hospital were placed in sterile tubes, washed with sterile saline to remove superficial debris and then dipped momentarily into boiling water to lessen surface-contamination. Scrapings were taken from the carious lesions and suspended in 0·5 ml. nutrient broth. (d) selected surfaces of teeth standing in the mouth were rinsed with sterile distilled water, dried with cotton rolls and the film on the tooth removed with a sterile scaler. This material was suspended in nutrient broth on a glass slide resting in a petri dish, using a technique designed to maintain asepsis. Each tooth-surface was examined three times at weekly intervals.

For primary isolation of "yeasts" tomato-peptone agar medium[3] was used and either (a) four drops of activated saliva delivered from a Pasteur pipette or (b) the material adherent to the swab or (c) and (d) the nutrient broth suspensions were distributed over the surfaces of the medium. The inoculated plates were incubated at 37° C. for 3–4 days.

After incubation the whitish conical colonies, 3–6 mm. in diameter, were picked from the tomato-peptone agar plates and subcultured onto 1 p.c. glucose agar slopes or into Robertson's cooked meat medium for storage.

Anderson's (1946) cornmeal extract medium was used to observe production of mycelium and chlamydospores. These features were examined microscopically in wet preparations and those strains which formed mycelium and chlamydospores were called *C. albicans*, the remainder yeast (including Cryptococcus).

The occurrence of lactobacilli in primary cultures on tomato-peptone agar was also recorded.

RESULTS.

Incidence of "Yeasts."

In the saliva. Although the majority of the "yeasts" isolated from the saliva were *Candida*, other genera can be found in this region. Considerable variation in frequency of occurrence has been reported by numerous investigators and this is probably due to the different methods used for isolating the organisms. Because both swab and saliva methods were used extensively in this study, a comparison of these methods was made. A group of boys (Boys' Town, Engadine) was examined firstly by swabbing the teeth and gums and then by collecting a sample of saliva. Of 109 only 37 p.c. of the group were found to harbour "yeasts" by the swab method in contrast to 47·7 p.c. by the saliva method. Another group of children (Bowral) was likewise examined by the two methods: the proportion showing "yeasts" were 11 p.c. and 20 p.c. by the swab and saliva method respectively.

Table 1 shows the results for several groups. It is interesting that the incidence of "yeasts" in samples of saliva from natives in New Guinea was similar to that in several groups of white Australians.

In carious teeth. Of the 64 teeth examined, 11 (17·2 p.c.) were found to have "yeasts" in the carious substance.

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[3] During the early part of this work a plate of tartaric acid 5 p.c. glucose agar was similarly inoculated. However, it was found that the tartaric acid medium did not reveal the presence of "yeasts" in any instance where it was not revealed by the tomato-peptone agar and, furthermore, that it hindered the growth of lactobacilli. Hence, as tomato-peptone agar supported the growth of both "yeasts" and lactobacilli it alone was used for primary isolation thereafter.

TABLE 1.

The incidence of "yeasts" in the mouth.

Group	Caries incidence	Method of sampling	Number in group	Number in group with yeast	P.c. in group with yeast
Children	high	swab	85	31	36·3
Prosthetic patients	—	swab	330	100	30·3
Students	high	saliva	207	93	44·9
Students	high	saliva	94	44	46·7
Students	high	saliva	186	63	33·9
New Guinea natives	very low	saliva	134	59	44·2
Boys' Town:					
1st examination	high	swab	108	40	37·0
1st examination	high	saliva	109	52	47·7
2nd examination	high	saliva	101	49	48·5
Bowral Children:					
1st examination	nil	swab and saliva	18	15	83·0
2nd examination	nil	swab and saliva	48	6	12·5
3rd examination	nil	swab and saliva	54	10	18·5
3rd examination	nil	swab	45	5	11·0
3rd examination	nil	saliva	45	9	20·0

TABLE 2.

The constancy of occurrence of C. albicans *in the mouths of individuals.*

Number of times C. albicans present	Number of Examinations I	II	III	IV	V	VI	VII
0	1	1			3	11	36
I						5	1
II		1			2		2
III						1	
IV				1		2	1
V					1	1	2
VI						1	5
VII							16

On tooth-surfaces. 33 tooth-surfaces were examined clinically and radiographically. Of these surfaces 19 were non-carious, 10 showed early carious lesions and 4 advanced lesions. Of the 19 non-carious surfaces six were covered by mucinous plaques containing "yeasts" but these organisms could not be isolated from any of the other surfaces.

Incidence of Candida albicans.

Constancy of occurrence in individuals. At monthly intervals for seven months samples of saliva from the same 94 students were examined for the presence of *Candida albicans*. The number of times the students were examined and the number of times *C. albicans* was found in the saliva are shown in Table 2.

Twenty students were found to be harbouring the organism each time they were examined, whereas 52 were found not to be harbouring *C. albicans* at any examination. In the saliva of the remaining 22 subjects the organism was found at some examinations but not at others. It might be noted that in 6 of these 22 subjects *C. albicans* was present at all but one examination, while in 6 others it was absent at all but one examination. The results indicate that the organism tends to be a constant inhabitant of some mouths and persistently absent from others. In only a few instances does it seem to be irregularly transient.

Constancy of occurrence in a group of individuals. The incidence of *C. albicans* in the group of 94 students was found to be 34·4 p.c. (mean of seven monthly examinations). The results are shown in Table 3 and it is obvious that there is no great variation. This necessarily follows the constancy of occurrence in the individuals comprising this group.

TABLE 3.

The group-incidence of C. albicans *in the same group of individuals examined over a period of 7 months.*

Month of year	No. examined	No. persons		P.c. persons +ve
		C. albicans +ve	*C. albicans*—ve	
May	82	26	56	31·7
June	84	31	53	36·9
July	88	31	57	35·5
August	88	30	58	34·1
September	89	30	59	33·7
October	87	30	57	34·5
November	84	29	55	34·5

Effect of presence or absence of teeth. Swabs from the mouth of a group of 209 edentulous individuals and a group of 121 partially edentulous individuals were examined for the presence of *C. albicans*. A comparison was made of the numbers of persons in each group who had *C. albicans* in their mouths and the value for χ^2 calculated (see Table 4). As this value was 0·659 no significant difference appears to exist between the incidence of *C. albicans* in these edentulous and partially edentulous groups.

If on the other hand a comparison is made of those individuals who are edentulous (i.e. have not worn an artificial denture after extraction of their teeth) and those who have teeth (either natural or artificial) the value for χ^2 is 12·0. This shows that the presence of teeth (natural or artificial) has a significant bearing on the incidence of *C. albicans* (see Table 4).

A similar relationship was found to exist for lactobacilli (Table 5).

Effect of "obvious dental caries." The term "obvious dental caries" is used to indicate that the degree of caries was sufficiently great to induce the patients to seek operative treatment. A control group consisted of individuals in whose teeth there were no untreated carious lesions and a few persons who were undergoing orthodontic treatment. However, the individuals in both groups were in all probability in a caries-susceptible state.

TABLE 4.

The effect of the presence or absence of teeth on the occurrence of C. albicans.

	No. persons		
	C. albicans +ve	*C. albicans* —ve	Total No. persons
Edentulous	47	162	209
Partially edentulous	32	89	121
Total	79	251	330

$\chi^2 = 0{\cdot}659$

	No. persons		
	C. albicans +ve	*C. albicans* —ve	Total No. persons
Completely edentulous	3	51	54
Persons with teeth or denture	76	200	276
Total	79	251	330

$\chi^2 = 12{\cdot}0$

TABLE 5.

The effect of the presence or absence of teeth on the occurrence of Lactobacillus.

	No. persons		
	Lactobacillus +ve	*Lactobacillus* —ve	Total No. persons
Edentulous	112	97	209
Partially edentulous	58	63	121
Total	170	160	330

$\chi^2 = 1{\cdot}0$

	No. persons		
	Lactobacillus +ve	*Lactobacillus* —ve	Total No. persons
Completely edentulous	11	34	45
Persons with teeth or denture	159	126	285
Total	170	160	330

$\chi^2 = 15{\cdot}3$

The results shown in Table 6 indicate that there is no relationship between "obvious dental caries" and the presence of *C. albicans*.

TABLE 6.

The relationship of obvious dental caries to the incidence of C. albicans.

	No. persons		
	C. albicans +ve	*C. albicans* —ve	Total No. persons
Persons with obvious caries	25	58	83
Persons without obvious caries	28	67	95
Total	53	125	178

$\chi^2 = 0{\cdot}008$

Effect of presence of lactobacilli. In view of the possibility of a symbiotic relationship between lactobacilli and *C. albicans* (Fosdick, 1937) the numbers of individuals with or without lactobacilli and with or without *C. albicans* were determined.

A value of 4·60 for χ^2 was found (Table 7). The presence of lactobacilli thus appears to be associated with the presence of *C. albicans* and vice versa.

TABLE 7.

The relationship between the occurrence of C. albicans *and of* Lactobacillus.

	No. persons		
	C. albicans +ve	*C. albicans* —ve	Total No. persons
Lactobacillus +ve	49	111	160
Lactobacillus —ve	30	120	150
Total	79	231	310

$\chi^2 = 4{\cdot}60$

TABLE 8.

The relationship of age to the incidence of C. albicans.

Group	Age range (years)	No. persons in Group	No. persons with *C. albicans*	No. persons with yeast	P.c. persons with *C. albicans*
A. Institution	1–4	25	2	3	8
B. Institution	3–4¼	18	3	12	16·6
C. Kindergarten	3–8	42	6	5	14·3
D. Students of Dentistry	19–24	94	31	13	34·4
E. Persons attending Prosthetics Dept., United Dental Hospital of Sydney	<25	32	7	21	21·6
	>25–<45	96	26		27·1
	>45	202	46		22·8

Effect of age and sex. Several groups of individuals were examined but, because the number of children examined was small, only tentative conclusions can be drawn from the results shown in Table 8. The incidence of *C. albicans* seems to rise from infancy to a maximum in early adult life.

A greater incidence in females than in males was found by Todd (1937); 18 p.c. in females and 9 p.c. in males. This observation is confirmed (Table 9) but the incidence reported here is greater than that given by Todd.

TABLE 9.

The occurrence of C. albicans *in males and females.*

	All ages	
	male	female
Number in group	87	241
No. persons *C. albicans* +ve	15	64
P.c. persons *C. albicans* +ve	17	26·6

DISCUSSION.

"Yeasts" from the mouth are a mixed group consisting mainly of species of the genus Candida, the remainder are ascosporogenous yeasts and cryptococci. The organism most frequently found is *Candida albicans* and it appears either to be a constant inhabitant in those individuals in whose mouths it is found or to be consistently absent. Hence the incidence in any one group of persons remains reasonably constant. The presence of teeth in the mouth is related to the presence of this organism so that, when the teeth are extracted, the organism disappears, but apparently returns when artificial dentures are worn. This sequence of events was found with lactobacilli also, a fact which had previously been established by American investigators.

C. albicans does not bear any constant relationship to dental caries but may play an important rôle as a symbiont. With this possibility in mind an investigation of the relation between lactobacilli and *C. albicans* was made. Although there is a significant association between their occurrence this may merely indicate a similarity in their physiological requirements and not necessarily a synergistic relationship.

The number of blastospores (yeast cells) in the mouth varies widely. Some of the New Guinea natives had counts as high as 500,000 cells/ml. White Australians usually had counts of less than 500 cells/ml.; some counts greater than 1,000 cells/ml. were however recorded.

Differences in incidence of "yeasts" appear to be partly explained by the use of different techniques for obtaining the samples of saliva. Collection of activated saliva was found both to be convenient and to show the highest incidence. However, for persons who had no teeth it was simpler to use the swab

method. It can be seen from the results that there is variation according to the method used.

Some workers claim a difference exists in incidence of yeast between caries-susceptible and immune individuals. The incidence of caries amongst New Guinea natives was considerably lower than that amongst students of dentistry, yet the incidence of yeast was very similar. Again, the incidence of caries among children (Bowral) was nil but the incidence of "yeasts" in these children was similar to that in a group of children who had a high incidence of caries.

The presence of "yeasts" as such would not appear to have any aetiological relation to the carious process. The fact that they are found in advanced carious lesions but not in plaques covering early carious lesions suggests that they are probably secondary invaders of the carious tooth—a suggestion in line with observations by others on the rôle of "yeasts" in dental caries.

SUMMARY.

"Yeasts" were observed in saliva of (a) children free from dental caries, (b) children with high incidence of caries, (c) three groups of students, (d) natives of New Guinea with a low incidence of caries and (e) boys at Boys' Town. They were also isolated from carious lesions and from tooth-surfaces (mucinous plaques).

The presence or absence of *Candida albicans* is a constant for any one individual.

The incidence of this fungus remains constant in any one group of subjects over a long period.

The organism disappears when all the teeth are removed but reappears when artificial dentures are worn.

No relationship between dental caries and *Candida albicans* was found.

Lactobacillus acidophilus and *C. albicans* occur together in the mouth and their association is statistically significant.

Variations in incidence of *Candida albicans* due to age and sex were observed.

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STUDIES OF THE FLORA OF THE MOUTH

IV. SOME OBSERVATIONS ON ACID PRODUCTION BY LACTOBACILLI AND CANDIDA ALBICANS: A PRELIMINARY REPORT

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The potential importance of synergistic actions between oral bacteria involved in the initiation of dental caries requires no elaboration. Hansen (1937) showed that a synergistic action occurred between *L. acidophilus* and yeast in the experimental dissolution of enamel in mixtures of glucose and enamel and of maltose and enamel. Fosdick (1937) found greater phosphatase activity but lower reductase activity in yeast than in lactobacilli. These observations established the facts on which he based his postulate of a symbiosis between the two organisms. Later, however, Knighton (1939) and Lilienthal and Goldsworthy (1949) showed that the majority of yeasts found in the mouth are *Candida albicans*.

The aim of the investigation reported briefly here was to examine some of the acid products of (a) aerobic and (b) anaerobic fermentation of glucose by *C. albicans* and by lactobacilli.

Materials and Methods.

Strains of *Lactobacillus acidophilus* and *Candida albicans* were isolated from the mouth on tomato-peptone agar medium and stored in Robertson's cooked meat medium (lactobacilli) or on glucose agar slopes (Candida). One strain each of a strongly acidogenic (SA) and a weakly acidogenic (WA) Lactobacillus (Sullivan, Still, Goldsworthy, 1939) and *C. albicans* were used.

Cultures of the organisms which had been incubated aerobically on glucose agar for 48 hours at 37° C. were washed once and then suspended in distilled water. Their dry weight was determined turbidimetrically after the instrument had been calibrated in turn for each organism.

The experiments were carried out in Thunberg tubes, with the exception that the aerobic fermentation of glucose by *C. albicans* was conducted in 100 ml. of 1 p.c. glucose broth in a

[1] This work was carried out while holding an Australian Dental Association, New South Wales Branch, and Walter and Eliza Hall Dental Research Scholarship of the University of Sydney.

flask from which, after incubation for 7 days at 37° C., samples were taken for the estimation of lactic acid (Barker and Summerson, 1941) and of volatile fatty acids (Friedemann, 1938). The Thunberg tubes contained 0·1 ml. of M/10 glucose, 1 ml. of the suspension of the organisms being tested and 0·4 ml. M/15 phosphate buffer. When mixtures of organisms were studied 1 ml. of each suspension was used. For aerobic studies air was the gas phase whereas for anaerobic conditions the tubes were thrice evacuated and filled with nitrogen passed over heated copper. After incubation of the Thunberg tubes for 1 hour at 37° C. sufficient trichloracetic acid was added to give a final concentration of 8 p.c. trichloracetic acid. Samples were then taken and the lactic acid-content determined. Control tubes were included in each series and all measurements were corrected accordingly.

RESULTS.

Aerobic fermentation of glucose by C. albicans. After 7 days' incubation at 37° C. only 1 millimole of volatile fatty acids but 1·3 gm. of lactic acid per litre were found. Both SA and WA lactobacilli exhibited the Pasteur effect.

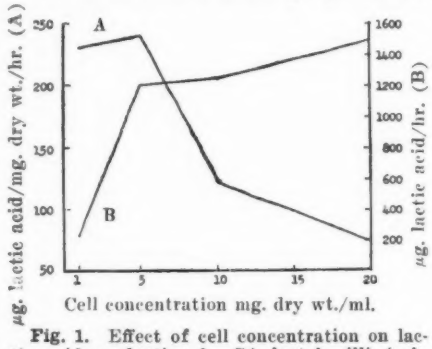

Fig. 1. Effect of cell concentration on lactic acid production by SA lactobacilli (substrate 0·1 ml. M/10 glucose).

Anaerobic fermentation of glucose. As the cell concentration of SA lactobacilli increased, the amount of lactic acid formed in the presence of M/15 phosphate buffer (pH 7·4) also increased. However, if the lactic acid production is determined for one unit of cell-substance, e.g. for one mg. dry weight, then it can be seen that the enzyme concentration reaches a maximum at approximately 5 mg./ml. dry weight (see Figure 1).

TABLE 1.

Effect of cell concentration on lactic acid production by SA lactobacilli (substrate 0·1 ml. M/10 glucose).

Cell concentration mg./ml.	microg/lactic acid/hr.	microg/lactic acid/mg. dry wt./hr.
1	231	231
5	1,191	238
10	1,231	123
20	1,491	75

Acid production experiments were performed with SA lactobacilli, WA lactobacilli, *C. albicans*, SA lactobacilli + *C. albicans*, WA lactobacilli + *C. albicans*, SA lactobacilli + heated *C. albicans* and WA lactobacilli + heated *C. albicans*. The results are set out in Table 2.

TABLE 2.

Microgrammes of lactic acid produced by lactobacilli and Candida *singly and in combination (incubated for 1 hour at 37° C.)*

pH	SA Lactobacillus	*Candida albicans*	SA Lactobacillus + *C. albicans*† (living)	SA Lactobacillus + *C. albicans*† (heated)*
6·5	65	12·0	160	65
7·0	65	7·5	100	205
pH	WA Lactobacillus	*Candida albicans*	WA Lactobacillus + *C. albicans*† (living)	WA Lactobacillus + *C. albicans*† (heated)*
6·5	81	8·0	72	236
7·0	74	7·5	70	226

* Cultures heated in boiling water for 30 minutes.

† The dry weight of *C. albicans* has been disregarded and the results are expressed in terms of mg. dry weight of lactobacilli.

The pH of the phosphate buffer had a marked effect on lactic acid production by SA and WA lactobacilli, but the results obtained have not been verified.

DISCUSSION.

The results presented, although only of a preliminary nature, indicate that some synergistic activity occurred between Lactobacilli and *C. albicans* under the conditions of the experiments. It is interesting that the so-called WA lactobacillus produced as much lactic acid in one hour with *C. albicans* as did the SA type. The increased lactic acid formation in the presence of heated *C. albicans* suggests that the sharing of enzymes is probably not the explanation of the increased acid-production. Some soluble compound, a vitamin or growth factor, amino acid or similar nutrient is more likely to be responsible for the effects observed.

The formation of lactic acid by *C. albicans* is not in agreement with the work of Van Niel and Cohen (1942) on the aerobic metabolism of this organism.

SUMMARY.

The lactic acid-production by SA and WA type lactobacilli and *Candida albicans*, singly and in combinations, has been studied. Synergistic effects were observed and an explanation is advanced in a brief discussion.

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THE FAILURE OF ANTIBODY PRODUCTION IN THE CHICK EMBRYO

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It is generally accepted that embryonic birds and mammals are incapable of producing antibodies and that the capacity to do so appears relatively soon after hatching or birth. The embryo whether *in utero* or *in ovo* is in a sterile environment well shielded from the entry of foreign material; in one way or another maternal antibody is made available for the passive protection of the newly born young and there seems to be neither biological necessity for, nor likelihood of, the precocious development of immunological reactivity in the embryo.

In mammals it is regularly found that very young individuals are poor antibody producers but the differences from the adult may not be as marked as was previously thought. In recent years it has become orthodox to immunize very young children against diphtheria and it is generally held that, provided no significant amount of maternal antitoxin is present in the infant's circulation, children of three months will give an effective antitoxic response (Glenny, 1949). Natural "antibodies", e.g. the isoagglutinins, do not appear until some weeks after birth. For obvious reasons very little has been done to determine whether antigens implanted in the mammalian foetus can provoke antibody. The only suggestion we have been able to find that foetal mammals can produce antibody is in a paper by Dettwiler, Hudson and Woolpert (1940). They inoculated guinea pig foetuses near term with influenza virus and showed that it multiplied in the lungs reaching maximum titres on the 6th day. Virus could not be demonstrated after 10 days and at this time antibody was present in both foetal and maternal blood, usually to a higher titre in the former. The question as to maternal or foetal production was left open. Even if, as the authors suggest, the antibody is produced by the foetus this may be an exception based on the high degree of development of the guinea pig at the time of birth.

Several attempts have previously been made to immunize chick embryos within the egg. Grasset (1929) found no response to diphtheria toxoid, the chicks when hatched being normally susceptible to the lethal action of toxin and having no circulating antitoxin. Burnet (1941) tested several chicks

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

hatched from eggs that had been infected with influenza virus and found no antibody. Fox and Laemmert (1947) made a more extensive investigation of the conditions with yellow fever virus infections of the chick embryo. Very few embryos survived unless the infection was initiated relatively late in the course of incubation. The majority of the chicks showed no antibody weeks after hatching but in a small proportion virus was detectable in the blood at the time of hatching and these subsequently developed antibody.

The present investigation was undertaken partly to test other antigens for their capacity to stimulate antibody in the chick embryo but primarily to determine whether administration of an antigen to the embryo interfered with subsequent capacity of the hatched chick to react to the same antigen.

In a recent review of the nature of antibody production, Burnet and Fenner (1949) have laid considerable stress on the probability that the mechanism, in virtue of which its own body components are non-antigenic for the individual concerned, is developed during the embryonic life of the individual. A brief statement of their hypothesis is necessary in order to explain the form taken by the experiments reported in this paper. It is axiomatic that although there is a rapid turnover of body components and, in the case of expendable cells like erythrocytes, disposal by a mechanism very similar to that which deals with foreign cells introduced artificially, such cells and components do not provoke antibody production. There must be some process by which the "scavenging" cells of the body can "recognize" body components as "self" and react to them in a different fashion from that appropriate to foreign, "not-self", components. It was asssumed that there is for each individual a limited number of what have been called self-marker components. One or more of these markers are present in each cell and there are corresponding mechanisms, "recognition units", within those cells, perhaps macrophages, in which the first stage of immunological response is initiated. The relation of these recognition units to the antibody production mechanism has been discussed in the monograph referred to but is not immediately relevant here.

Some evidence was adduced to suggest that these recognition units were not determined solely by genetic factors but resulted from the exercise of the process of disposal of expendable cells during embryonic life. It was thought possible that any potentially antigenic "pattern" constantly present during embryonic life would permanently condition the scavenging cells against immunological response to this particular pattern. The most interesting example where such a mechanism may be functioning is to be found in Owen's demonstration of "erythrocyte mosaic" in calves from certain multiple births (Owen, 1946). Where twin calves are of binovular origin and develop an anastamosing placental circulation haemopoietic cells appear to be freely interchanged. As a result each of the twin calves may show red cells of two antigenic types, one corresponding to its own genetic type, the other to that of its twin. The coexistence of the two types persists for life.

Traub (1939) described a mouse colony in which almost all individuals were infected with the virus of lymphocytic choriomeningitis *in utero* and if they were born alive carried through life a heavy generalized infection with the virus without showing symptoms or producing antibody. Normal animals artificially infected with the same strain showed clinical illness and after recovery were free of the virus and partially immune. A challenge inoculation of virus intracerebrally might produce symptoms but after recovery from this, immunity was firm and complement fixing antibody detectable in the blood.

It was thought that by previous administration of representative antigens to the embryo it might be found possible to demonstrate an altered reactivity in the hatched chick to subsequent immunization with the same antigen. The experiments gave no positive findings but it seemed advisable to place a brief account of the results on record.

Techniques.

Our basic technique was to inject a suitable potential antigen into the embryo by an appropriate route, allow the embryo to hatch and when the chick was 5–8 weeks old test its serum for antibody and then establish its capacity to respond to an immunizing infection of the same antigen. Various modifications of this plan were introduced according to circumstances.

The following antigens were used:

Living influenza A virus. It was found that practically no chicks hatched successfully with standard strains well adapted to allantoic passage. A recent 1948 strain, FJS, and a substrain of Melbourne (MEL) which had undergone only one or two chick embryo passages were therefore used. The embryos were infected by allantoic inoculation and multiplication verified by testing a sample of allantoic fluid for haemagglutinin after 3 days' incubation at 35° C. The eggs were then returned to the stock commercial incubator at 38° C. and allowed to hatch.

Bacterial virus C16. This is a standard strain used extensively by Burnet (1933) and antigenically closely related to the T2 phage of Luria and Delbrück. It was grown on the dysentery strain Y (Hiss and Russell). In some experiments another phage, Asheshov's K of the same serological group, was used. The embryos received 1 ml. of phage (clarified broth culture heated to 56° for 30 minutes, having a titre of 1×10^{10} plaque-producing units per ml.) injected into the yolk sac.

Human red cells. These were washed cells made up to a 50 p.c. suspension in saline. For intravenous inoculation 0·05 ml. was used, 0·5 ml. for yolk sac inoculation.

Serological methods. Chickens were bled from the wing vein and the serum separated and inactivated at 56° C. before testing.

Antibody against influenza virus was titrated by antihaemagglutinin tests against the homologous virus. The technique was that in general use in this laboratory using a final 0·5 p.c. concentration of fowl cells and 5 agglutinating doses of virus. The endpoint of partial agglutination was determined when the cells had settled at room temperature.

Phage inactivating antibody was titrated by Burnet's (1933) method in which serial dilutions of serum are mixed with phage diluted so as to give, when a mixture with inert serum is plated on the susceptible bacterial strain, about 100 plaques from the standard volume of 0·02 ml. The serum-phage mixtures were kept at 37° C. for 1 hour before being plated. The only modification of the original technique was to use the serum dilution producing 50 p.c. reduction in plaques as the endpoint instead of the 20 p.c. previously adopted.

Human cell agglutinins were tested at room temperature. Serial dilutions in saline of the serum received an equal volume of a 1 p.c. suspension of washed cells and the usual endpoint of partial agglutination was taken.

EXPERIMENTAL.

(1) *Behaviour of Chicks Inoculated* in ovo *with Influenza Viruses.*

In the first experiment with the recent A strain, FJS, three chicks hatched successfully from a batch of sixteen inoculated at 11 days by the allantoic route. All had shown positive haemagglutination by a sample of allantoic fluid taken 2 days after inoculation. The embryos inoculated into the yolk sac at 8 days all died with the exception of two which hatched successfully. (Since these had shown no haemagglutinin in a sample of yolk fluid it is probable that they had not been infected.) All five chicks were bled at 5 weeks after hatching and showed no antibody.

At 6 weeks these with two control chicks of the same age were inoculated intramuscularly with 1 ml. of FJS allantoic fluid, the inoculation was repeated a week later and serum tested after a further lapse of 10 days. The first section of Table 1 shows the results obtained.

It will be seen that an effective antibody response was obtained in all chicks, the differences shown being probably insignificant.

TABLE 1.

Antibody response to influenza virus of six week old chicks previously infected in ovo *with influenza virus.*

Chick No.	Treatment in ovo			Antibody response at 6 weeks		
				Anti-haemagglutinin on day		
	Virus	Route	Proof of Infection	0	3	17
42	FJS	Allantoic	+	<10		110
45	FJS	Allantoic	+	<10		70
51	FJS	Allantoic	+	<10		70
49	FJS	YS	?	<10		240
50	FJS	YS	?	<10		240
24	Nil	—	—	<10		120
100	Nil	—	—	<10		120
80	MEL	Allantoic	+	<10	640	
83	MEL	Allantoic	+	<10	960	
89	MEL	Allantoic	+	17	1,100	
93	MEL	Allantoic	+	<10	560	
105	Nil	—	—	<10	320	
106	Nil	—	—	<10	640	

A more extensive experiment carried out on the same lines with a substrain of MEL which had undergone only one preceding allantoic passage gave eleven chicks, all from embryos with proved infection following allantoic inoculation at 11 days. These were bled six weeks after hatching and four were found to show small amounts of antibody in their serum, their anti-haemagglutinin titres being 50, 17, 17, 15. The other seven had no detectable amount. Four chicks, three with no antibody and one with a small amount, were inoculated intravenously with 1 ml. of homologous virus in the form of MEL allantoic fluid. Two controls were similarly treated. Other experiments had by this time shown the extreme rapidity with which anti-

influenza virus antibody appears after intravenous injection in the chick and in order to detect any evidence of delay in antibody production serum samples were obtained 72 hours after the immunizing injection. As will be seen from Table 1 there was again no significant difference amongst the birds tested.

(2) *Behaviour of Chicks Inoculated* in ovo *with Bacterial Viruses.*

Embryos were inoculated in the yolk sac at 6 days with 1 ml. of a lysed culture heated to 56° C. for 30 minutes and containing 1×10^{10} virus particles (plaque count) per ml. Two chicks treated *in ovo* with C16 and six with K were available. At 6 weeks none showed any anti-phage activity. In one group of sera merthiolate to 1:10,000 was added through a misunderstanding. This made it impossible to obtain satisfactory bacterial growth in mixtures with undiluted sera. In Table 2 the serum samples concerned are shown as having a titre below 0·5. Sera indicated as having zero titre allowed a full plaque count in mixtures in which the final dilution of serum was 1 in 2. Intravenous injection at six weeks of 1 ml. of the homologous bacterial virus gave the antibody responses shown in Table 2.

TABLE 2.

Response to intravenous injection of bacterial virus of chicks previously treated in ovo.

Chick No.	Treatment	Antiphage titre on day shown				
		0	3	4	5	7
210	C16 YS	0		1·1		2·5
211	C16 YS	0		0		1·5
240	Nil	0		2·3		2·9
241	Nil	0		1·5		2·6
108	K YS	<0·5	0·6		2·3	
110	K YS	<0·5	1·5		2·6	3·0
114	K YS	<0·5	1·9		3·0	
116	K YS	<0·5	1·0		2·4	
201	Nil	0	1·5			2·4
202	Nil	0	0·6		2·2	2·0

Note that titres are expressed logarithmically, 2·0 for instance indicating that serum diluted 1:100 produced a 50 p.c. reduction in plaque count under standard conditions.
YS. Yolk sac inoculation of 6-day embryos.

Except in the case of one chick (211) there is again no suggestion that treatment *in ovo* has any influence on subsequent immunological reactivity.

(3) *Reactivity with Human Red Cells.*

Normal fowl serum agglutinates human red cells to a variable titre, the following titres being obtained from twelve adult white leghorn fowls, 160, 130, 130, 120, 120, 100, 60, 30, 30, 15, 10, 10.

Chicks under six weeks contain no agglutinin and most of them develop detectable amounts within the next four weeks. In view of the similarity of this situation to the appearance of isoagglutinins in human beings an experiment was carried out to determine whether administration of human cells *in ovo* might prevent the subsequent development of the natural agglutinin. A group of twenty 11-day embryos was inoculated intravenously with 0·05 ml. of a 50 p.c. suspension of washed human cells and twelve of these hatched. Sera were taken from

six weeks onward and tested for agglutinin against human red cells with the results shown in Table 3. A similar experiment was carried out in which a larger amount of antigen, 0·5 ml. of 50 p.c. red cells, was injected into the yolk sac of twelve 6-day embryos. Eight of these hatched and six survived to the completion of the experiment. It will be seen that between the 6th and 10th weeks all chicks in both groups developed agglutinin apparently somewhat more rapidly than normal chicks.

TABLE 3.

Proportion of chicks at different ages showing serum haemagglutinin for human cells.

Treatment in ovo	Ages in weeks						
	6	7	8	9	10	11	12
Human cells intravenously	1/12	4/12	6/10	8/10	10/10		
Human cells in yolk sac	1/6	4/6	4/6	4/6		6/6	
Nil	0/11		2/10	1/6	2/6	4/6	6/6

Number of sera showing titre of 10 or more, over number tested.

DISCUSSION.

These experiments confirm earlier findings that material which is highly antigenic for 6–9 week chicks is incapable of producing antibody when injected into the embryo. The occasional exception observed with living influenza virus corresponds with Fox and Laemmert's (1947) similar results with yellow fever virus. They found that in some chicks at the time of hatching living virus was still present and that only these subsequently developed detectable antibody. In our own experiments no tests for virus were made at the time of hatching but it seems reasonable that, in some, virus had persisted into the period at which the chick was developing immunological reactivity.

The work was undertaken primarily to see whether experience of an antigen in embryonic life would render the organism subsequently incapable of responding to that antigen. The work of Owen and Traub previously referred to, suggested that this might be possible. If so, a very potent approach to immunological theory would become available. No evidence of such an effect has, however, been disclosed by the present experiments. This may mean that the whole conception of the development of self-marker recognition during embryonic life is wrong but it is at least as likely that the process requires the continued presence in the embryo of the potential antigen from a very early stage. This requirement is fulfilled by the foreign erythropoietic tissue in Owen's work and the lymphocytic choriomeningitis virus in Traub's but not in our own experiments. It may be possible to find a virus which, like lymphocytic choriomeningitis virus in the mouse, can produce general infection without death in very young chick embryos but none of the influenza viruses we have tested will do so.

It should be emphasized that for the antigens with which we have been concerned the fowl gives an extremely rapid antibody response after intravenous injection and is in many ways a most suitable object for the study of basic immunological phenomena.

SUMMARY.

Injection of foreign red cells, bacterial virus or living influenza virus into chick embryos did not provoke antibody production.

No change in the subsequent response of the hatched chick to the corresponding antigen could be demonstrated.

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THE HAEMOLYTIC ACTION OF NEWCASTLE DISEASE VIRUS

I. THE TWO TYPES OF INTERACTION BETWEEN VIRUS AND RED CELL

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In the course of work on haemagglutination by the viruses of mumps and Newcastle disease of fowls it has often been noted that haemolysis occurs to a greater extent than is observed with influenza viruses. Incidental experiments made in the past showed that the haemolysis was very incomplete and that many infected fluids failed to show any such activity. The stimulus to investigate haemolysis by Newcastle disease virus (NDV) arose in part from the publication of the paper by Morgan, Enders and Bagley (1948) on mumps virus haemolysis, and in part from the observation that when cells were treated by NDV in phosphate solution preparatory to electrophoretic studies by Mr. G. L. Ada, much more extensive haemolysis occurred than in physiological saline.

While the work was in progress the first report on haemolysis by NDV to appear in the literature (Kilham, 1948) reached Australia. Kilham's paper covers many of the points which had been established in our own work and of which a brief account had been submitted for publication (Burnet, 1949). It has seemed desirable, however, to present a comprehensive account of the interesting and complex phenomena presented by the virus in its relation to red cells and to ovomucin and other virus inhibitors. In the present series of papers it will be shown that haemolysis by NDV is mediated through the same receptor mechanism as is concerned with haemagglutination, and that it appears to be intimately associated with the capacity of the virus to become irreversibly attached to the cell. The present communication is concerned mainly with a re-examination of the general characteristics of NDV interaction with fowl and human erythrocytes.

In 1946 it was shown that under suitable conditions both NDV (Burnet and Anderson, 1946) and mumps virus (Burnet, 1946) could be firmly attached to red cells so that the stabilized cells were agglutinated by specific immune sera. Anderson (1947) made a detailed study of the conditions with NDV. He established conclusively that the agent responsible for the characteristic

1 This work was carried out under a Grant from the National Health and Medical Research Council, Canberra.

2 This is the paper referred to as "Burnet (1950)" in Burnet and Lind, this Journal, 28, p. 129.

behaviour of "sensitized" cells was the virus particle itself. In the course of this work he showed that the stabilized cells could agglutinate normal cells and that there was a characteristic extension of haemagglutinin titre when virus and red cells were allowed to interact for some time at 37° C. This phenomenon he ascribed to disaggregation of the virus. The work to be reported confirms that of Anderson in all essentials, but is largely concerned with the circumstances under which NDV, acting on red cells, behaves like an influenza virus in not giving any firmly attached virus. It is possible to define the conditions under which the two different types of reaction occur, and the recognition of the characteristics of the two types of action is essential to the understanding of the haemolytic reactions.

Materials and Methods.

Newcastle Disease Virus.

The Australian strain of Albiston and Gorrie (1942) has been used exclusively. All stocks are prepared by allantoic inoculation of 11-day chick embryos with 10^{-4} dilution of seed virus stored with 50 p.c. glycerol at —10° C. Fluid is harvested after 42–44 hours' incubation at 35° C. from chilled eggs.

Virus was used either in the form of allantoic fluid or after partial purification and concentration by one of two methods. "Cold eluate" was prepared by adsorbing virus on to washed fowl cells at 0° C. and washing the cells in cold saline. The cells were then suspended in half the original volume of saline and the virus allowed to elute for 18–20 hours at room temperature (17–20° C.) Methanol-precipitated virus was prepared by Mr. G. L. Ada using a modification of the method of Cox *et al.* (1947). This is described elsewhere (Burnet and Lind, 1950).

Other virus strains referred to are the standard strains used in this Institute and have frequently been described.

Haemagglutination Tests.

Washed fowl cells from birds known to provide cells satisfactory for inhibitor titrations and not agglutinated by lipid suspensions were used. The final dilution in the tests was 0·5 p.c. of cells and the readings were made to a standard (one plus) pattern of partial agglutination. Dilutions were made in 0·85 p.c. saline and read after settling at room temperature. In some experiments Anderson's technique of subsequently transferring the racks to 37° C. and observing the agglutination patterns after one or more re-settlings at that temperature was used. Human cells, Group O, were used similarly when required.

The presence of "fixed" (irreversibly bound) virus on treated cells was determined by the use of immune fowl serum prepared by Anderson (1947) and lyophilized. The reconstituted serum diluted 1:100 gives strong agglutination of cells carrying fixed virus. The more quantitative test called by Anderson ASC titration (agglutinin on stable cells) was also used. In this a 1 p.c. suspension of the cells to be tested is diluted serially in normal saline. To each tube an equal 0·25 ml. volume of a 1 p.c. suspension of normal fowl cells is added and the agglutinin titre read in the usual fashion after settling at room temperature.

Other Techniques.

The methods used in measuring haemolytic activity and electrophoretic mobility will be described in other papers. Only incidental reference is made to these results in the present communication.

Reactions carried out at specified temperatures were done in a water bath thermostatically controlled and constant to ±0·25° C.

When cells coated with fixed virus were required the usual procedure was to add 0·25 ml. of packed cells to 5 ml. of allantoic fluid virus of haemagglutinin titre 250–500. After 1 hour at 37° C. the cells were washed twice with saline and made up to 1 p.c. suspension in saline. In some experiments cells were treated with a preparation of the *V. cholerae* enzyme RDE after the action of NDV (30 minutes at 37° C. with final concentration of about 100 RDE units in buffered Ca saline).

EXPERIMENTAL.

The Influence of Temperature on the Reaction of NDV with Red Cells.

In practically all previous experiments on cells stabilized after virus action the reaction was carried out at 37° C., and the characteristics of such cells have been described in previous papers. The fact that under suitable conditions haemolysis occurs at 37° C. but not at room temperature (17–18° C.) prompted a closer study of cells treated with NDV at the lower temperature. It was found that under these conditions cells could be stabilized only after long exposure and then irregularly. The unstable cells could, however, be stabilized with a very dilute immune serum. This differentiates them immediately from cells treated with the virus at 37° C. These are agglutinated by immune serum. If cells stabilized by long exposure (overnight) to virus at room temperature or by the addition of small amounts of immune serum with subsequent washings, are tested for susceptibility to agglutination by our standard series of viruses, susceptibility is normal for all viruses except mumps and NDV. Unstable cells produce some agglutination when mixed with normal cells but when stabilized either spontaneously or by immune serum have no such action. The electrophoretic mobility of cells treated under different conditions will be described in detail by Mr. G. L. Ada; here it is only necessary to state that the cells treated at 17° C. have a mobility of 0·7–0·9 (μ/sec./volt/cm.) as against 0·4–0·5 for those treated at 37° C. (Normal cells 1·3). Table 1 gives a summary of the properties of such cells in contrast to those treated under similar conditions at 37° C.

TABLE 1.

Properties of human red cells treated to equilibrium with NDV.

	Treated at 17°	Subsequent RDE	Treated at 37°	Subsequent RDE
Haemolysis	—	•••	+	•••
Spontaneous agglutination	++	—	—	—
Agglutination by immune serum	— (Stabilization)	—	++	++
Agglutination by I–M serum	No Stabilization	—	++	++
ASC titre	(8)	—	20	20
Agglutination by NDV	—	—	—	—
Agglutination by MEL	++	—	—	—
Agglutination by WSE	++	—	+	—
Agglutination by LEE	++	—	++	—
Electrophoretic mobility (μ/sec./volt/cm.)	0·76–0·81		0·43–0·56	

I–M Serum. Serum from case of infectious mononucleosis.

Apart from the degree of haemolysis produced, the various characteristics shown in Table 1 are independent of the type of virus preparation used provided it has not been damaged by heat or chemical manipulation. The most clear cut and easily demonstrable difference between the two types of cell is their reaction to a Newcastle disease immune serum. Cells treated at room temperature are not agglutinated and if initially unstable are stabilized, cells treated at 37 °C. are strongly agglutinated. It is convenient to refer to the 17 °C. cells as Type 1 and the 37° C. cells as Type 2.

When cells are tested at various intermediate temperatures it is found that the temperature at which the change from Type 1 to Type 2 occurs is higher for fowl than for human cells.

TABLE 2.

Effect of NDV on cells at different temperatures.

Temperature	Fowl cells		Human cells	
	N.S.	I.F. S/100	N.S.	I.F. S/100
20	+	—	++	—
22			++	—
24			—	++
26	—	—	—	++
28	—	±	—	++
31	—	++	—	++
37	—	++	—	++

N.S. Normal saline. I.F. S/100 1:100 dilution of immune fowl serum. Degree of agglutination of cells is shown.

In Table 2 the time of exposure at different temperatures varies considerably as the experiments were concerned primarily with haemolysis and samples were taken earlier at the higher temperatures. However, with the amounts of virus used the characteristic change in all characters took place very rapidly and incidental findings in many other experiments have always been in conformity with the tabulated results. As is shown in another paper of this series (Burnet and Lind, 1950) haemolysis with suitable virus only becomes evident at and above the temperature, 29° C. for fowl cells, 24° C. for human cells, at which the change-over to Type 2 reaction occurs. No significant changes in the results were observed at temperatures up to 50° C.

Changes Produced on Heating NDV in Phosphate Buffer.

It was noted by Anderson (1947) that heating to 56° C. left the haemagglutinating activity of NDV almost unaltered but that the virus eluted much more slowly. A more detailed study of the influence of heat on the virus has been made in which virus in the form of allantoic fluid was diluted 1:5 in M/15 phosphate buffer pH 7·35 and heated for 30 minutes over the range 50°–58° C. at 2° C. intervals. For each sample the haemagglutinin titre, haemolytic power and enzymic activity on ovomucin were titrated, and a test of infectivity made using material undiluted and diluted 1:100. Fowl cells were treated at 37° C. for 4 hours with each sample and, after washing, tested for stability, ASC titre, agglutination or stabilization by

immune serum, and loss of receptors to the viruses used in defining our standard receptor gradient. The results are shown in Table 3. The points to be noted are (1) the loss between 52° C. and 54° C. of haemolytic activity, of power to stabilize cells and to give evidence of firmly attached virus on the cells; (2) the persistence of haemagglutinating activity to 58° C. with infectivity and enzymic activity becoming greatly decreased at that temperature.

TABLE 3.

Activity of NDV heated in phosphate (pH 7·35) for 30 minutes at different temperatures.

Temperature	H.A.	Infectivity	Haemolysis	Enzymic action	Fowl cells		F4/100	Mu	ND	M	WS	L
					Stability	ASC						
Unh.	640–1,280	$>10^2$	++	120	—	10	++	—	—	—	+	++
50	360–480	$>10^2$	++	120	—	3·5	++	—	—	+	++	++
52	360–480	$>10^2$	+	120	—	1—	+	—	—	+	++	++
54	360–400	$>10^2$	—	120	+	1—	—	—	—	++	++	++
56	320–320	$>10^2$	—	320	++	(2)	—	—	—	++	++	++
58	320–320	10^0	—	2,500	++	(2·5)	—	—	—	++	++	++
No virus	—	—	—	10,000	—	—	—	++	++	++	++	++

Notes: ***H.A.*** Haemagglutinin titre at first reading and highest subsequent reading after 4 shakings and resettlings. ***Enzymic action.*** Level to which inhibitory titre of ovomucin reduced after treatment overnight at 37° C. with 20 agglutinating units. Indicator T-MEL.

Gradient tests. Mumps, Newcastle disease, Melb, WS and LEE viruses 5 AD's were used. Results with virus heated 54°–58° C. obtained after preliminary stabilization of cells with immune serum.

Human cells behaved in almost exactly the same fashion as fowl cells. In a subsequent experiment virus was heated under the same conditions to 52° C., 54° C. and 56° C. in order to examine the changes induced in the electrophoretic mobility of human red cells treated with virus.

TABLE 4.

Action of heat on NDV as tested with human cells.

Temperature	H.A.	Haemolysis	Treated human cells		F4/100	MU ND M WS	Electrophoretic Mobility (μ/sec./volt/cm.)
			Stability	ASC			
Unh.	800	++	—	20	++	— — — +	0·45
52	640	+	—	8	±	— — ± ++	0·57
54	400	—	+	(2)	—	— ± ++ ++	0·88
56	480	—	++	(3)	—	— — ++ ++*	0·91
No virus	—	—	—	—	—	++ ++ ++ ++	1·30

* Cells stabilized with dilute immune serum before being tested.

The results (Table 4) confirm those of the previous experiments and in addition show a marked difference in electrophoretic mobility which is correlated with the other types of change in the cell surface produced by the virus. The significance of the changes in electrophoretic mobility which are induced by virus action will be discussed in a forthcoming paper by Ada and Stone.

Here it need only be noted that NDV at 37° C. has a much greater effect on the charge of the red cell than would be expected from the limited extent to which receptors are removed.

In a further paper the enhanced haemolytic activity of NDV precipitated by methanol in the cold will be described. Such a preparation in phosphate-buffered saline was heated to 52° C. 54° C. and 56° C. The changes in haemagglutinating and haemolytic action on red cells and in the enzymic action on ovomucin were precisely similar to those shown in the preceding Tables. Haemolysis was produced by 52° C. sample, not by those heated at 54° C. or 56° C.

In summary it may be said that by heating virus to 54° C. for 30 minutes NDV loses only those additional properties that distinguish its *in vitro* behaviour from that of a normal influenza virus, viz. its power to coat cells irreversibly with virus, to lower the electrophoretic mobility to a value around 0·45 and to haemolyse. The suggestion therefore arises that these three actions are due to a single additional property of the virus.

Quantitative Aspect of the Two Types of Reaction with Fowl Red Cells.

The full character of cells with firmly attached virus is only seen when there is a relatively high virus/cell ratio. The following experiment is typical of several. Mixtures of washed fowl cells at final concentrations of 4, 2, 1 and 0·5 p.c. were made with serial twofold dilutions of virus (red cell "cold" eluate). Portions of each mixture were transferred to another set of tubes to observe the changes in haemagglutination on repeated shaking and the main set then placed in a water bath at 37° C. for 1 hour. The tubes were then shaken, centrifuged, the supernatants separated and the cells washed with saline and made up to a 1 p.c. suspension. The fluid was tested for free virus and the cells examined for stability in saline, susceptibility to agglutination by NDV and by immune fowl serum and for their ASC titre with fowl cells.

TABLE 5.

Analysis of reaction between NDV and fowl cells for 1 hour at 37° C.

	Final dilution of virus									
	6·2	12·5	25	50	100	200	400	800	1,600	3,200
4 p.c. Free virus	+	+	+	+	—	—	—	—	—	—
Agglutinable cells	—	—	—	—	—	+	+	+	+	+
ASC, I.S.	16+	7·5+	3·5+	<2—	—	—	—	—	—	—
2 p.c. Free virus	+	+	+	+	+	—	—	—	—	—
Agglutinable cells	—	—	—	—	—	—	+	+	+	+
ASC, I.S.	16+	11+	8+	2+	<2	—	—	—	—	—
1 p.c. Free virus	+	+	+	+	+	+	—	—	—	—
Agglutinable cells	—	—	—	—	—	—	—	+	+	+
ASC, I.S.	16+	12+	7+	5+	2·5±	2—	—	—	—	—
0·5 p.c. Free virus	+	+	+	+	+	+	±	—	—	—
Agglutinable cells	—	—	—	—	—	—	—	—	—	+
ASC, I.S.	16+	14+	15+	8+	3+	<2±	—	—	—	—
1 p.c. Agglutination RT	++	++	++	++	++	++	++	+	—	—
1 p.c. Agglutination 37° C.	—	—	—	—	—	—	+	++	+	—

+ Following ASC titre indicates that cells were agglutinated by immune fowl serum (I.S.).

The abbreviated summary in Table 5 establishes a number of interesting points. In the first place it is clear that each type of action is dependent on the virus/cell ratio. Destruction of agglutinability by NDV proceeds to a much higher dilution than the full development of ASC and the related characteristics. A considerable amount of virus fails to reappear in the supernatant fluid, the amount, as would be expected, being roughly proportional to the concentration of cells.

TABLE 6.

Successive treatment of NDV dilutions with fresh fowl cells.

Initial virus dilution	Free virus (A.D.)				ASC			Immune serum			Agglutination by NDV		
	0	I	II	III	0	I	II	0	I	II	0	I	II
25	27	20	18	9	11	5	1·5	++	++	—	—	—	—
50	14	10	8	1	6·5	(—)	(—)	++	—	—	—	—	—
100	7	4·5	—	—	2·2	(—)	<1	—	—	—	—	—	++
200	3·5	1·5	—	—	<2	<1	<1	—	—	—	—	++	++
400	1·7	<1	—	—	<2	<1	<1	—	—	—	—	++	++

Note: ASC titres marked (—) indicate that cells were unstable and as a result showed some agglutination with normal fowl cells.

Further information can be gained from Table 6 giving the results of a similar experiment using a final concentration of 1 p.c. fowl cells throughout. Virus dilutions in 5 ml. volumes were allowed to react with cells for 1 hour at 37° C., the cells and supernatant fluids were separated by centrifugation and tested as in the previous experiment. The greater part of the supernatant fluid was then returned to the 37° C. bath and packed fowl cells added to give a 1 p.c. suspension. After an hour the process was repeated.

The most important point to note in the Table is the changing ratio between the amount of virus initially present as judged by its haemagglutination titre and the ASC titre acquired by the cells exposed to it. With each exposure to cells there is a much greater decrease in the capacity to "coat" the cells than in the overall haemagglutinin titre.

Extension of NDV Haemagglutinin Titre at 37° C.

In Anderson's (1947) paper a good deal of stress was laid on the evidence that NDV existed largely in the form of virus aggregates. Electron micrographs which have since been published, Dawson and Elford (1949), Bang (1948), give no positive evidence of the existence of such aggregates. It is therefore desirable to re-examine the evidence for Anderson's conclusion. Essentially this was based on the fact that when the cells in a haemagglutinin titration series were allowed to settle at 4° C. or 20° C. and, after reading, reshaken and transferred to 37° C., the next reading showed a significant increase in apparent titre. Anderson postulated primary agglutination by aggregates bridging from one cell to another. With shaking in the presence of cells these aggregates were broken up and could then show activity to a higher dilution.

A number of observations, some due to Anderson and others made in the course of the present work, pointed toward the simpler explanation that at 37° C., given sufficient time, dilute virus might eventually take on the irreversible type of union which would provide a stronger binding force between erythrocytes than the type obtaining at 20° C. and below. The points in favour of this view are (1) the necessity of a temperature at or near 37° C. to demonstrate the increase; (2) the parallelism between what Anderson called CSA titre (i.e. the dilution to which the virus would under standard conditions change the cells to give the characteristic agglutinability by immune serum) and the haemagglutinin titre at 37° C.; (3) the fact that virus heated to 54° C. or higher fails to show the increase in titre at 37° C. and, (4) that ASC titrations also show an increase in titre at 37° C.

Two experiments may be presented. The first whose results are given in Table 7 is representative of several. It is an attempt to analyse the conditions actually present in the mixtures showing the appearance of agglutination at 37° C. after being unagglutinated at 20° C. After agglutination had occurred with three successive reshakings the cells and supernatant fluids were separated. The fluid was tested for free virus by its power to agglutinate fresh fowl cells. The cells (fowl) after washing with saline were tested for (1) stability (2) ASC and agglutinability by immune serum and (3) for susceptibility to agglutination by 5 AD's of NDV.

TABLE 7.

Analysis of extension of titre shown on reshaking at 37° C.

	Virus dilutions							
	100	2	4	8	16	32	6,400	Titre
First reading	++	++	+±	±	—	—	—	600
Fourth reading	—	—	+±	+±	±	—	—	1,200
Supernatant	++	—	—	—	—	—	—	
Cells stability	—	—	±	—	—	—	—	
Cells agglutination by NDV	—	—	+	++	++	++	++	
Cells agglutination by ASC	+	—	—	—	—	—	—	
Cells agglutination by F7	±	—	—	—	—	—	—	

Degree of haemagglutination shown by conventional symbols.

It will be seen that over the zone concerned with the extension of titre (1) the cells are almost completely stabilized on washing; (2) there is no free virus in the supernatant; (3) the cells are agglutinable by NDV. The inference is that in these tubes a relatively small proportion of cells carry irreversibly bound virus particles and produce what is essentially ASC agglutination of the majority of normal cells.

In the course of work on the haemolytic action of virus firmly held to stromata, the capacity of the coated stromata, subsequently treated with RDE and washed several times, to agglutinate normal fowl cells was tested at 20° C. and after reshaking at 37° C. Three types of stromata were used: (1) Stromata resulting from lysis of fowl cells by NDV after RDE treatment gave titres 20° C., 90; 37° C., 130; (2) Stromata produced by saponin lysis of fowl

cells, thoroughly washed and treated with methanol-precipitated virus at 37° C., then with RDE gave titres of 20° C., 8; 37° C., 32; (3) Red cells treated with NDV allantoic fluid to give NDV-F cells, subsequently lysed with saponin and washed gave titres 20° C., 28; 37° C., 70.

It is almost inconceivable that aggregates could have been present on these stromata throughout the numerous manipulations and the electron micrographic evidence is directly opposed to it.

Further discussion of this phenomenon is more conveniently deferred to the general discussion.

DISCUSSION.

These results should be considered in relation to Anderson's (1947) previous work in which a full study is reported of the standard procedure of treating cells with active allantoic or amniotic fluid virus at 37° C. The present results confirm his findings in regard to such action. The main interest of the present work is its demonstration that under appropriate conditions another type of reaction of virus with red cells can occur which is entirely comparable to the action of the influenza viruses. If active virus is used at a temperature below 30° C. for fowl cells or 24° C. for human cells adsorption of virus, agglutination, elution of virus, destruction of receptors and complete or partial stabilization occur without any of the special features of Newcastle disease virus action at higher temperatures. These are (1) irreversible adsorption of a proportion of virus particles to the cell surface with the resultant phenomena, capacity to agglutinate normal cells, agglutinability by specific immune serum and by many sera from patients with infectious mononucleosis; (2) haemolysis and (3) unduly great reduction in electrophoretic mobility. The second and third features will be discussed in subsequent papers.

The present discussion will be confined to the relation between the two types of virus-cell interaction that have been described. The type of reaction which is seen below the critical temperature, at near limiting dilutions of active virus or with virus heated to 54° C., is clearly analogous to the standard type of influenza virus action. This will be referred to as Type 1 action. In this the virus particles are adsorbed to the cell receptors presumably by an "active group" which either is, or contains as an essential part, the enzyme which rapidly destroys the specific character of the receptor so that the union is released. It is possible that the virus particle is held to the cell surface by more than one mutual interaction between virus and cell surface components so that a single enzymic action may not liberate the virus particle. But in any case the process of repeated adsorption and release goes on until cell receptors capable of adsorbing the virus are destroyed. Macroscopic agglutination occurs as long as the conditions are such that there are a sufficient number of virus particles held by significant adsorptive forces to the surface of two cells so

that they can form a "bridge" between them. Agglutination fails on the one hand when the receptors remaining are too few in number or activity and, on the other, when the number of virus particles is too few to provide an adequate number of "bridges" to allow macroscopic agglutination.

The irreversible union Type 2 does not occur with cells that have been treated with RDE or viruses to the right of NDV in the gradient series but once it has occurred it is not dissociated by RDE or by immune serum. From experiments of the type shown in Tables 5 and 6, as well as from Anderson's previous results, it is clearly necessary to postulate heterogeneity of the virus population. The simplest hypothesis is that all virus particles in a natural preparation, not unduly exposed to damaging influences, have approximately equal activity for Type 1 reaction with the cell receptors, but that their capacity to give rise to firm Type 2 union differs widely. We assume that the population of virus particles could theoretically be divided into a series of component populations each of uniform individuals. At one end of the series the virus particles will produce firm union once in every x effective contacts with cell receptors while at the other end of the series firm union will result only once in nx contacts. There are no data to offer any quantitative estimate of the values of x or n, but it is likely from experiments of the type illustrated in Table 6 that the limiting value nx may be very high. In nearly all experiments where conditions near the virus/cell ratio corresponding to the agglutination endpoint were being studied, one mixture could be found in which all demonstrable NDV receptors had been removed from the cells but no free virus was detectable. This is compatible with the view that the majority of virus particles can eventually reach a firm union with some cell receptor. It is obvious that cells will only show the characteristic behaviour of cells carrying fixed virus when there is an adequate number of firmly fixed virus particles on each cell. When the ratio of Type 2 : Type 1 contacts is so low that several cells will be "stripped" of receptors for each firm union established, the only evidence of the existence of these unions that could be expected would be an extension of apparent titre at 37° C. This would result from the greater "bridging" capacity of virus particles with at least one firm union to a cell as compared to those making transient Type 1 unions only. This could be manifested only in mixtures where susceptible cells persisted after the effective completion of all possible virus action.

No evidence has yet been obtained to indicate in what respects the physicochemical character of Type 2 union differs from the normal process that is common to all the viruses of the group.

SUMMARY.

Newcastle disease virus, NDV, has two characteristic modes of action on fowl or human erythrocytes.

Type 1 action corresponds to that of most influenza viruses, reversible adsorption associated with enzymic destruction of receptors and elution. This is shown to varying extent under all the conditions which allow recognizable interaction between virus and cell surface and is the only type shown below 25° C. or by virus heated between 54° C. and 58° C.

Type 2 action, irreversible union of virus to the cell receptors, is observed only with active unheated virus, acting on cells at a temperature above 24° C. (human cells) or 28° C. (fowl cells). The same conditions are necessary for the manifestation of three other actions of NDV on red cells, haemolysis, abnormal reduction in electrophoretic mobility and extension of haemagglutinin titre at 37° C.

The phenomenon of extension of titre at 37° C. described by Anderson is probably due not to disaggregation but to the gradual development of Type 2 virus-cell unions.

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ENZYMATIC MECHANISMS IN THE RESPIRATION OF RHUBARB LEAVES. Part 2.

by J. F. MORRISON

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(Accepted for publication 19th December, 1949.)

In a previous paper (Morrison, 1949), a preliminary survey of the metabolic reactions of rhubarb leaves was reported together with evidence which indicated the presence of cytochrome oxidase or a similar enzyme in the leaf. This paper further deals with the effect of inhibitors and substrates on the rate of respiration together with a more detailed investigation of aconitase.

Methods.

Respiration was measured by means of Warburg manometers and the tissue was prepared as previously described (Morrison, 1949).

The Action of Inhibitors.

Fluoride Inhibition.

The respiration of rhubarb leaves was found to be inhibited to a large extent by fluoride, in concentrations of 1 mg./ml. and 0·1 mg./ml., the CO_2 liberating mechanism being more sensitive (Table 1).

TABLE 1.

Fluoride inhibition of leaf respiration.

Treatment	1st hour		2nd hour		3rd hour		Inhibition 3rd hour	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
Control	51	56	43	48	49	55	—	—
1 mg./ml. F	15	0	9	0	7	0	86 p.c.	100 p.c.
0·1 mg./ml. F	11	8	12	7	14	6	72 p.c.	86 p.c.

Fluoride, although not completely specific, is very often associated with the inhibition of enolase, which converts 2-phosphoglycerate to pyruvate through phosphoenolpyruvate. In order to determine if this were the site of action, pyruvate and glucose were added to the tissue poisoned by fluoride, whence it was found that there was no increase in the oxygen uptake at pH 5·3. However, at pH 7·0 there was a slight increase in the oxygen uptake by pyruvate, although at the same time there was a decrease in the percentage inhibition (Table 2).

TABLE 2.

Effect of glucose and pyruvate on fluoride inhibition at pH 7·0.

Treatment	O_2 uptake in mm.^{3}		
	1st hour	2nd hour	3rd hour
Control	150	126	109
Control + fluoride	100	78	62
Control + F + P.A.	123	93	74
Control + F + glucose	100	82	60

F = fluoride. P.A. = pyruvic acid.

Pyruvate could increase the O_2 uptake by approximately 20 p.c., but glucose had no effect upon the inhibited rate of O_2 uptake. It seems therefore that fluoride at least partially inhibits a process involving the conversion of glucose to pyruvic acid. Pyruvic acid has been isolated from barley by James and James (1940) and from onions by Bennett (1945). The failure of pyruvate to affect the inhibition at pH 5·3 can be explained as the failure of penetration, whereas at pH 7·0 it is at least partially able to diffuse into the cell. The fact that the inhibition is not completely lifted may be due to the inhibitory action of fluoride on other oxidative systems. Results do, however, point to the possibility of the presence of enolase, and thus of the initial oxidation of carbohydrates by means of phosphorylation.

Malonate Inhibition.

The respiration of rhubarb is inhibited by malonate, the degree depending both on the concentration of the inhibitor and the pH of the medium. (Table 3).

TABLE 3.

Inhibition by malonate at various pH values.

pH	4·5		5·3		6·3	
Treatment	O_2	CO_2	O_2	CO_2	O_2	CO_2
Control	130	158	109	114	123	135
5 mg./ml.	38	41	49	57	78	71
(Inhibition)	(70 p.c.)	(79 p.c.)	(55 p.c.)	(50 p.c.)	(37 p.c.)	(48 p.c.)
1 mg./ml.	76	70	92	100	105	98
(Inhibition)	(42 p.c.)	(50 p.c.)	(16 p.c.)	(12 p.c.)	(15 p.c.)	(27 p.c.)

The decrease of inhibition could be explained either by the fact that at higher pH values, the cell is less permeable to malonate because of its ionic structure, or by the assumption that malonate exerts its maximum inhibitory action only when the first carboxyl group is ionised. In animal tissues malonate inhibits whilst doubly ionised, so if the site of inhibition is the same, the former would be the more likely explanation.

Quastel and Wooldridge (1928) have shown that malonate is a specific inhibitor of succinic dehydrogenase in bacteria, and it has since been taken that the inhibitory action of malonate on respiration is indicative of the presence of succinic dehydrogenase. In the presence of large amounts of malonate the inhibition of the oxygen uptake was never greater than about 80 p.c., which suggests that respiration through succinic dehydrogenase accounts for only

four-fifths of the total respiration. The simultaneous inhibition of the CO_2 evolution by approximately the same amount suggests that succinic dehydrogenase is also a part of the CO_2 liberating mechanism.

The Effect of Organic Acids on Malonate Inhibition.

The overcoming of malonate inhibition of this enzyme by the structurally related succinic acid and the similar effect of fumarate and the other intermediate tricarboxylic acids, which were oxidatively converted to succinate, was an important piece of evidence in the development of the tricarboxylic acid cycle (Krebs, 1943). On infiltrating succinic, fumaric and malic acids into the leaf cell at pH 5·3, in the presence of malonate at a concentration of 5 mg./ml., it was found that succinate and malate were at times able to overcome the malonate inhibition. These irregular results seem to be related to the condition of the leaf. It was found that malonate had no inhibitory action on a very mature leaf, so it appears that there is a change in the metabolism of rhubarb leaves during the course of maturity. These preliminary results do not give any indication whether a tricarboxylic acid cycle exists in the leaf at any stage of growth.

Iodoacetate Inhibition.

The respiration of rhubarb leaves was found to be strongly inhibited by iodoacetate. (Table 4.)

TABLE 4.

The inhibition of respiration by iodoacetate.

Treatment	1st hour		2nd hour		3rd hour		Inhibition 3rd hour	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
Control	55	63	70	81	67	77	—	—
10^{-2} M I.Ac.	4	1	11	0	9	0	87 p.c.	100 p.c.
10^{-3} M I.Ac.	19	23	20	13	11	5	84 p.c.	93 p.c.

I.Ac. = iodoacetate.

It can be seen that the enzyme mechanisms of rhubarb leaf are very sensitive to iodoacetate inhibition, a ten-fold decrease in the concentration of the inhibitor causing only a small decrease in the percentage inhibition.

Iodoacetate is by no means a specific enzyme inhibitor, although it is known to inhibit enzymes such as triose dehydrogenase and malic dehydrogenase. The fact that iodoacetic acid is a powerful inhibitor of rhubarb leaf respiration does not present evidence of the presence of these dehydrogenases, although it gives a general indication. The dehydrogenases of the leaf will be determined at a later date.

The Metabolism of Starved Leaves.

The Effect of Starvation on Metabolism.

Freshly picked leaves do not appear to be limited in their rate of respiration by substrate concentration. Therefore, in order to determine the effects of organic acids on the respiration, leaves were starved by placing them in a dessicator containing a strong aqueous solution of sodium hydroxide and stored in a dark room.

To determine what period of dark storage in a CO_2-free atmosphere was required for a marked fall in the rate of respiration, leaves were stored and samples taken after 24, 48 and 72 hours. Table 5 shows the effects of starvation.

TABLE 5.

The effect of starvation on leaf respiration.

(measured during the 2nd hour.)

Period of starvation	O_2 uptake	CO_2 output	R.Q.	Dry weight	QO_2
nil	115	152	1·31	65 mg.	1·77
24 hours	123	159	1·29	70 mg.	1·76
48 hours	64	82	1·29	99 mg.	0·65
72 hours	26	15	0·58	164 mg.	0·16

In all starvation experiments it was noted that there was a marked fall in the O_2 uptake and CO_2 output after 48 hours' storage, but the R.Q. value did not decrease until after 72 hours' storage.

Accompanying the fall in respiration, there was a noticeable yellowing of the leaf. These leaves have presumably been depleted of carbohydrate reserves.

The Results of Substrate Additions to Starved Leaves.

It was found that glucose, citrate, *cis*-aconitate, α-ketoglutarate, succinate, fumarate, malate and pyruvate were able to stimulate the respiration to some degree, when infiltrated into starved leaves, so it can be assumed that these acids are normal cellular components. The CO_2 production was more markedly stimulated than the O_2 uptake by all the substrates added, with the exception of glucose (Table 6).

TABLE 6.

The effect of glucose and organic acids on the respiration of starved leaves.

Substrate added	Concentration	P.c. increase in mm.^{3}		R.Q.	
		O_2	CO_2	Control	+ substrate
Glucose	10 mg./ml.	22	11	0·94	0·86
Succinate	5 mg./ml.	18	46	0·94	1·18
Fumarate	5 mg./ml.	13	40	0·94	1·16
Malate	10 mg./ml.	—	40	0·94	1·34
Pyruvate	10 mg./ml.	11	46	1·09	1·27
Citrate	5 mg./ml.	—	22	1·09	1·47
cis-Aconitate	5 mg./ml.	15	19	1·09	1·13
α-Ketoglutarate	5 mg./ml.	20	22	1·09	1·10

Malate and citrate were at no time able to stimulate the rate of oxygen uptake although they did increase the CO_2 production.

Glutamic Decarboxylase.

Schales, Mims and Schales (1946) have discovered a glutamic acid decarboxylase in a variety of higher plants. Okunuki (1937) found that this enzyme could not be brought into solution and concluded that it was bound to the cell walls. However, the Schales and Mims (1946) found that it was possible to prepare clear solutions of the enzyme from carrots.

Rhubarb leaves were found to contain a very active glutamic acid decarboxylase. This discovery was accidental. It was noted that on adding glutamate to respiring leaves the CO_2 production was many times greater than the O_2 uptake, which suggested that a decarboxylation reaction was taking place. The rate of reaction was determined at pH 5·3 using glutamate in a concentration of 3·3 mg./ml. It was found that the activity of the enzyme could be demonstrated using both discs and homogenates.

The Activity of Discs and Homogenate in N_2 and Air.

In order to compare the rates using discs and homogenate, the leaf was homogenised in a Waring blendor with an amount of phosphate buffer (pH 5·3) for 2–3 minutes, so that 2·5 ml. of the homogenate were equivalent to 400 mg. leaf. Table 7 shows a comparison of the results obtained. Both nitrogen and air were used as the gas phase, but there was little difference in the rate of decarboxylation, although in air there was an extra uptake of oxygen.

TABLE 7.

Comparison of the rates of decarboxylation in nitrogen and air.

Gas phase	Material	1st 30 mins.	2nd 30 mins.	3rd 30 mins.	4th 30 mins.
Nitrogen	discs	155	88	35	33
	homogenate	148	92	18	12
Air	discs	134	78	36	36
	excess O_2	2	6	6	10

The results indicate that there is little difference in activity using different methods. In general, however, it was noted that the decarboxylation using the homogenate was more liable to variation. There was a rapid loss of enzyme activity after the first 30 min., presumably caused by the inhibitory nature of the product produced as a result of decarboxylation.

The clear supernatant liquid obtained on centrifuging the homogenate was found not to possess any activity which suggests that the decarboxylase is bound to the cell wall.

The effect of dialysis. On dialysis of the homogenate for 24 hours it was found that there was a large loss of activity, which points to the fact that a dialysable prosthetic group is required for activity. The Schales and Mims (1946) have shown that pyridoxal phosphate is able to restore the decarboxylase activity of carrots after dialysis and it therefore seems quite likely that the same prosthetic group is required by the rhubarb glutamic decarboxylase.

The effect of inhibitors. Cyanide at concentrations of 10^{-2} M and 10^{-3} M caused a 100 p.c. inhibition of the enzyme, doubtless by forming a cyanhydrin complex, whilst fluoride (1 mg./ml.) inhibited the rate of decarboxylation by 30 p.c. Iodoacetate had no effect on the activity.

Aspartic decarboxylase. Tests were carried out to determine the presence of aspartic acid decarboxylase, but there was no evidence for the existence of this enzyme in rhubarb.

Trans-Aconitic Acid Inhibition.

Trans-aconitic acid has not been previously used as an inhibitor of metabolism, the reason being that its site of action is unknown. It has, however, been reported by McCalip and Seibert (1941) to occur in plants, but it seems most likely that the *trans* acid was derived from the *cis* form as the method of identification involved the drying of leaf samples at 80° C., a temperature at which *cis*-aconitic acid can be readily converted to the *trans* form.

Krebs (1943) has suggested that *trans*-aconitic acid may be inhibitory in the respiration of pigeon breast muscle and that it is for this reason that *cis*-aconitic acid is in equilibrium with citric acid, although the latter lies outside the direct pathway of metabolism. If such was not the case, then, when the rate of *cis*-aconitate formation was greater than its utilisation, inhibitory *trans*-aconitic acid could be formed.

The Effect of Trans-*Aconitic Acid on Respiration.*

Trans-aconitic acid was found to inhibit the respiration of rhubarb leaf discs (Table 8).

TABLE 8.

Inhibition of respiration by trans-*aconitate.*

Treatment	1st hour		2nd hour		3rd hour		Inhibition 3rd hour	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
Control	112	117	100	121	83	97	—	—
10 mg./ml.	59	50	49	38	38	22	54 p.c.	77 p.c.
5 mg./ml.	71	64	59	55	50	41	40 p.c.	58 p.c.
1 mg./ml.	64	53	58	45	51	40	40 p.c.	59 p.c.

It can be seen that the CO_2-liberating mechanisms are more sensitive to *trans*-aconitate and that there is no difference in the percentage inhibition by concentrations of 5 mg./ml. and 1 mg./ml.

The Effect of Citrate and cis-*Aconitate on* trans-*Aconitate Inhibition.*

Citrate and *cis*-aconitate at concentrations of 10 mg./ml. and 5 mg./ml. were found to have no effect on the inhibition of respiration by *trans*-aconitate (5 mg./ml.). This may mean that *trans*-aconitic acid does not inhibit the aconitases, but rather has some other site of action, or, that the inhibition is irreversible and not competitive.

At pH 7·0 it was found that *trans*-aconitic acid was without effect on the respiration so presumably this acid is unable to penetrate the cell wall at this pH.

The finding that *trans*-aconitic acid was inhibitory to cellular respiration prompted the investigation to determine if the aconitases were present in the leaf, and if so, whether they were inhibited by *trans*-aconitic acid.

Aconitase.

Jacobsohn and Soares (1940) have reported the presence of aconitase in the seeds of cucumber, pumpkin and peas, and in the stem, leaf and root of the cabbage. They showed that this enzyme could be divided into two fractions, α-aconitase which brings about the conversion

cis-aconitic acid $\rightleftharpoons$ isocitric acid

and β-aconitase which converts

cis-aconitic acid $\rightleftharpoons$ citric acid.

Berger and Avery (1943) have assumed that active aconitase must be present in the Avena coleoptile, as citric acid was rapidly oxidized by partially purified extracts, whilst Bonner and Wildman (1946) have also assumed the presence of aconitase in spinach leaves, as a result of the oxidation of citric acid.

Material and Methods.

A β-aconitase was found to occur in the leaves of rhubarb as shown by the ability of a phosphate buffer extract to convert *cis*-aconitic acid to citric acid and *vice versa*. Maximum extraction was obtained with 0·1 M phosphate buffer of pH 7·2 as determined by the Thunberg tube technique using citrate as substrate, together with Coenzyme II, and 2:6 dichlorphenol indophenol as an electron acceptor. The extract was obtained by grinding the leaf with three volumes of buffer, incubating one hour at 37° C., then squeezing through muslin and centrifuging. The extract was green and opaque.

Cis-aconitic acid was prepared by the method of Anschutz and Bertram (1904). Its estimation was carried out by the method of Johnson (1939) which, although not specific for *cis*-aconitic acid, was sufficiently accurate when a blank determination was also carried out. Citric acid was determined according to Krebs and Eggleston (1944), using hydrogen peroxide as a decolorising agent for potassium permanganate in place of ferrous sulphate.

Aconitase Activity.

To test the activity of the enzyme, 11·2 mg. of *cis*-aconitic acid were incubated under anaerobic conditions with 5 ml. of the extract and aliquots taken at intervals. Table 9 shows the results of this experiment.

TABLE 9.

The rate of conversion of cis*-aconitate by aconitase.*

Time (min.)	0	20	40	60	80	100
Cis-aconitic acid remaining (mg.)	11·2	3·91	2·42	1·15	0·56	0·30

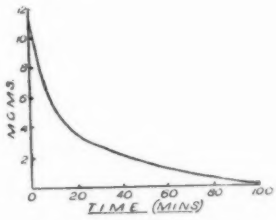

Fig. 1. Illustrating the rate of conversion of *cis*-aconitate by aconitase.

By graphing these results (Fig. 1) it can be seen that the action of β-aconitase conforms to that of usual enzyme systems.

The Equilibrium between cis*-Aconitic and Citric Acids.*

The equilibrium between *cis*-aconitate and citrate under anaerobic conditions was found greatly to favour the formation of the latter. When the concentration of *cis*-aconitate added was greater than 0·004 M, 85 p.c. was converted to citrate, whilst below this concentration the percentage conversion was less (Table 10).

The same amount of citrate remained when citrate was used as a substrate, as was formed when *cis*-aconitate was added. The amount of *cis*-aconitate remaining after incubation is small and possibly the failure to establish *cis*-aconitate as a plant acid is due to this fact. In all experiments a loss of acid was noted and this may be due to the conversion to isocitric acid. If such is the case, then the equilibrium mixture of the tricarboxylic acids in the rhubarb leaf is similar to that which occurs in pigeon breast muscle.

TABLE 10.

Citrate formed and cis-*aconitate remaining after incubation of the extract with* cis-*aconitate.*
(Incubation time, 180 min.)

Aconitate added (mg.)	Molarity	Citrate formed (mg.)	P.c. conversion	Aconitate remaining (mg.)	P.c. remaining	P.c. loss
1. 5·625	0·001	1·54	28	—	—	—
2. 11·25	0·002	7·06	63	—	—	—
3. 22·5	0·004	19·85	85	1·29	6	9
4. 45·0	0·008	38·21	85	2·06	5	10
5. 67·5	0·012	57·04	85	3·35	5	10
6. 90·0	0·018	76·39	85	5·21	5	10
7. 112·5	0·022	91·71	82	—	—	—

The Action of trans-*Aconitic Acid on Aconitase.*

The structural similarity of *trans*-aconitic acid, the ready conversion of one form to the other and the inhibitory action of the *trans* acid on the overall respiration, prompted the investigation of the effect of this acid on the attainment of the equilibrium between *cis*-aconitic acid and citric acid by aconitase. Table 11 shows that *trans*-aconitic does inhibit the action of the enzyme.

TABLE 11.

Inhibition of the conversion of cis-*aconitate to citrate and citrate to* cis-*aconitate by* trans-*aconitate.*

Cis-*aconitate.*

Conc. of *trans*-aconitate	Amount *cis*-aconitate added	Amount citrate formed	P.c. conversion	P.c. inhibition
M/50	22·5 mg.	7·63 mg.	34	62
M/100	22·5 mg.	8·53 mg.	38	55

Citrate.

Conc. of *trans*-aconitate	Amount citrate added	Amount citrate remaining	P.c. loss	P.c. inhibition
M/50	22·5 mg.	21·73 mg.	4	75
M/100	22·5 mg.	21·53 mg.	5	72

This particular reaction is at least one site where the *trans*-aconitic acid is able to inhibit, but it is possible that it is also able to inhibit other enzymatic reactions.

DISCUSSION.

The study of rhubarb leaf metabolism by the measurement of gas exchanges after various treatments of the leaf, shows that in some respects the enzymatic reactions which occur are similar to those of animal tissues and some plants.

The inhibition of respiration by fluoride and the ability of pyruvate, but not glucose partially to overcome the inhibition, points to the fact that carbohydrate may, to some extent, initially undergo degradation by means of phosphorylation. If such is the case, then the end product of the phosphorylative mechanism, from what is known of animal metabolism, would be pyruvate. Starved leaves are able to utilize pyruvate, so the production of this acid is quite possible.

A mechanism for the further oxidation of pyruvate is the tricarboxylic acid cycle. In this mechanism, an acetyl derivative of pyruvate condenses with oxalacetate to form *cis*-aconitate which is converted to isocitrate. Isocitrate then undergoes oxidation and decarboxylation through α-ketoglutarate, succinate, fumarate and malate to oxalacetate. This series of reactions accounts for the aerobic oxidation of carbohydrates in a number of animal tissues.

The ability of rhubarb leaves to utilize the organic acids which are components of this system, is at least circumstantial evidence in favour of the existence of the cycle. This idea is supported by the presence of aconitase and succinic dehydrogenase, two enzymes which play an important rôle in the cycle, but the irregular results obtained from the addition of organic acids to malonate-poisoned leaves cannot be taken as evidence for or against the cycle. A comparison of the general reactions of rhubarb and spinach leaves, the Avena coleoptile and animal tissues, shows that there is a close similarity in their metabolism, except that spinach leaves contain polyphenol oxidase, whereas the other tissues contain cytochrome oxidase.

Moulds are also able to produce and utilize a number of the organic acids of the tricarboxylic acid cycle as well as acetone dicarboxylic, glycollic, glyoxalic, gluconic and saccharic acids. However, although plant metabolism has been suggested to be similar to mould metabolism, there has been no general approach to the investigation of mould metabolism as there has been in the case of the three plants mentioned; so a comparison of the metabolic behaviour of rhubarb leaves and moulds is not possible.

In the formulation of any system to explain the oxidation of carbohydrates by rhubarb leaves, the occurrence of large amounts of oxalate cannot be overlooked. Oxalate also occurs in high concentrations in spinach leaves, but no attempt has yet been made to determine the origin of this acid. To date, this specific problem has not been studied in the case of rhubarb, but it is interesting to note that two possible precursors of oxalate are oxalacetic and glyoxalic acids.

In animal tissues succinic dehydrogenase is able to transport electrons more or less directly to the cytochrome system. The presence of both succinic dehydrogenase and cytochrome oxidase indicates that the succinic dehydrogenase of the leaf is similar to that which occurs in wheat and animal tissues.

SUMMARY.

Fluoride and iodoacetate markedly inhibited the respiration of rhubarb leaves. The addition of pyruvate at pH 7·0 partially overcame the fluoride inhibition.

Respiration was inhibited by higher concentrations of malonate. The inhibition was not decisively overcome by fumarate, malate or succinate.

Glucose, succinate, fumarate, malate, pyruvate, citrate, *cis*-aconitate and α-ketoglutarate stimulated the respiration of starved leaves.

An active glutamic decarboxylase occurs in the leaf. It was inactivated by dialysis and inhibited by cyanide and iodoacetate.

Trans-aconitate inhibited the respiration of leaf discs, also the activity of the β-aconitase which occurs in the leaf.

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THE HYDROGENASE OF *E. COLI* IN THE CELL-FREE STATE

I. CONCENTRATION, PROPERTIES AND ACTIVATION

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The nature of the enzyme hydrogenase has been the subject of controversy ever since its discovery. It has been studied mainly in *Escherichia coli* in which it was discovered (Stephenson and Stickland, 1931), *Azotobacter vinelandii* (Phelps and Wilson, 1941; Wilson *et al.*, 1942) and in *Proteus vulgaris* (Hoberman and Rittenberg, 1943; Farkas *et al.*, 1947). The assumption so far has been that the enzyme possesses the same basic properties in all three groups of organisms; and although this has so far been borne out in a number of respects, there are some discordant results.

Stephenson and Stickland classed hydrogenase as a dehydrogenase type of enzyme, for, although they found that carbon monoxide inhibited to a certain extent, cyanide had no effect. Wilson and co-workers, interested in the rôle of hydrogenase in nitrogen fixation, continued the study on inhibitors; but neither cyanide, nor hydroxylamine, fluoride nor iodoacetate affected the enzyme.

Hoberman and Rittenberg (1943) followed hydrogenase activity by studying the "exchange reaction," i.e.

$$D_2 + H_2O \rightarrow D_2O + H_2; \quad D_2 + H_2O \rightarrow HDO + HD;$$

or more simply $H_2 \rightarrow 2H^+ + 2e$.

The basic assumption is that while both in this reaction and in the reduction of methylene blue only hydrogen is enzymically activated, the latter might involve unknown carriers or electron donors, while the reaction studied here is presumably direct. Hoberman and Rittenberg found that cyanide inactivated hydrogenase *completely* under aerobic conditions, without having any effect under anaerobic ones. Oxygen inhibited it completely and reversibly; carbon monoxide inhibited strongly. They concluded that the hydrogenase of *Proteus vulgaris* was an iron-porphyrin enzyme. Farkas *et al.* repeated and extended this work and reached the same conclusion, while Waring and Werkman (1944) showed that *Azotobacter indologenes*, grown on a medium deficient in iron, lacked hydrogenase in addition to several enzymes which are known to contain iron (catalase, peroxidase, etc.).

Very little work has been done on hydrogenase in the cell-free state, the only communication being a short note by Back *et al.* (1947). These authors crushed cells of *Escherichia coli* in a ground glass mill and examined the effect of certain inhibitors on the enzyme both under "aerobic" and "anaerobic" conditions. Cyanide and hydroxylamine inhibited under the former but had no effect under the latter conditions.

In this paper this work on cell-free hydrogenase which supplies the most direct evidence of the nature of the enzyme is continued and extended.

[1] This work was carried out during the tenure of a Commonwealth Junior Research Studentship during 1948.

Materials and Methods.

The strain of *E. coli* which was used in this work was the American strain B, kindly supplied by Dr. M. Delbrück. The medium used was: 1 p.c. glucose, 1 p.c. Gurr's peptone, 0·5 p.c. sodium chloride, dissolved in M/15 phosphate buffer pH 7·6. The bacteria were grown in amounts of up to thirty litres at 37° C. for 18 to 36 hours, and then collected by centrifuging at 40,000 r.p.m. in a Sharples supercentrifuge. Hydrogenase activity was estimated by measuring the rate of hydrogen uptake in Warburg manometers. The gas phase consisted of hydrogen which had been passed over copper turnings heated to 400° C., and the hydrogen acceptor was methylene blue (0·4 ml. of a 2 p.c. solution per cup). If inhibitors were used, they were, unless otherwise stated, added to the enzyme before gassing. Since it was found that cell-free preparations did not evolve carbon dioxide, no alkali was used in the centre well. The volume was always made up to 3·0 ml. by adjusting the amount of 0·2 M phosphate buffer pH 7·6, which was used throughout. Thioglycollic acid was used in the free state, since in the amounts used its effect on the pH was negligible.

Reagents used were generally the purest available, and solutions of them were kept at 5° C. A number of them, like sodium hydrosulphite, ammonium polysulphide and very dilute solutions of ferrous sulphate were always made up immediately before use.

EXPERIMENTAL.

Preparation of the Cell-Free Extract.

The bacteria, grown and collected as described above, were mixed with alkali-free powdered glass and the resulting paste crushed in a glass-mill according to the method of Kalnitsky *et al.* (1945). Glass-distilled water was found to be the best medium for crushing, as it gave more active enzyme preparations than 0·2 M phosphate buffer of pH 8·0, 7·6 or 6·0, or 0·1 M potassium chloride. It was found generally that passing the bacteria through the mill three times broke them up to such an extent that maximum extraction of the enzyme in a "soluble" form, (i.e. at which the enzyme activity is not deposited after half an hour's centrifuging at 4,500 r.p.m.) was obtained. After each passage the paste was diluted about one in ten with distilled water, and centrifuged to deposit all glass and bacterial particles. The resulting enzyme solution was pale yellow and only slightly opalescent. The hydrogenase activity was consistently high and kept for well over four months. The activities of different solutions of the enzyme which are referred to as ES (e.g. ES 1a, ES 1b, ES 2, etc.), are always expressed in µl. hydrogen absorbed per ml. per hour.

TABLE 1.

Activities of certain hydrogenase preparations.

(Activities expressed as µl. hydrogen/ml./hr.)

Enzyme preparation	Activity
After crushing the first time and eluting (ES 1a)	17,000
After crushing the second time and eluting (ES 1b)	6,960
After crushing the third time and eluting (ES 1c)	4,880

Properties.

A knowledge of the working properties of hydrogenase in ES 1 enabled a scheme of purification to be finally worked out.

Heat Resistance.

If a small amount of ES 1 is immersed in a boiling water bath till a certain temperature is reached, and is then plunged into an ice-salt mixture, so that the total time above 50° C. is not more than 60 seconds, a considerable amount of inactive material coagulates, but the hydrogenase activity is only slightly diminished. The smaller the volume of enzyme being heated, the smaller is the loss in activity. When, however, 300 ml. of the enzyme preparation are to be thus treated, a compromise must be made, and ten ml. portions heated to between 50° and 60° C. were found to be satisfactory.

TABLE 2.

Effect of heat on hydrogenase.

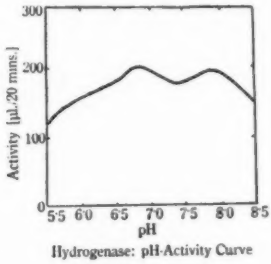
Treatment	Activity in µl./ml./hr.
—	105
70° C. in 3 ml. portions	80
50° C. in 10 ml. portions	61
50° C. in 20 ml. portions	50
3 ml. boiled for one minute	40

pH.

The pH curve for the enzyme is shown in Fig. 1. Two maxima are discernible.

Dialysis.

Dialysis tends to inactivate hydrogenase owing to oxidation. The more purified the enzyme preparation used, the more pronounced is this effect. It can be prevented by the addition of a few drops of 0·1 M thioglycollic acid to the dialysing liquid. There is no dilution effect.



Hydrogenase: pH-Activity Curve

Fig. 1.

Organic Solvents.

Absolute alcohol and dioxane inactivate the enzyme completely, but, as Bovarnick (1941) has shown, acetone precipitates the enzyme without destroying the activity. The best results are obtained if one to two ml. of ES 1 are added to five to six volumes of acetone at —15° C. and the mixture is centrifuged quickly. There should be no flocculation of the precipitate, and the supernatant after centrifuging should still be a little milky. Under those conditions acetone destroys little hydrogenase activity even if acetone containing up to 10^{-4} M hydrochloric acid is used. With larger volumes the losses in activity rise steeply.

Precipitation with Ammonium Sulphate.

Hydrogenase is almost completely precipitated by two-thirds saturated ammonium sulphate, but very little is precipitated by one-twelfth saturation, which, however, precipitates a large amount of inactive protein and other enzymes.

Extraction of Hydrogenase from the "Residues."

Even after the bacteria have been crushed three times, a certain amount of cells and/or cellular fragments remains behind, and was found to be rich in the enzyme. The residue after one-sixth saturation with ammonium sulphate of ES 1 also contained hydrogenase (15 to 25 p.c. of the amount in ES 1), and it was found that an appreciable amount of the enzyme

went into solution from both these residues if they were treated with alkaline phosphate buffer pH 8·0.

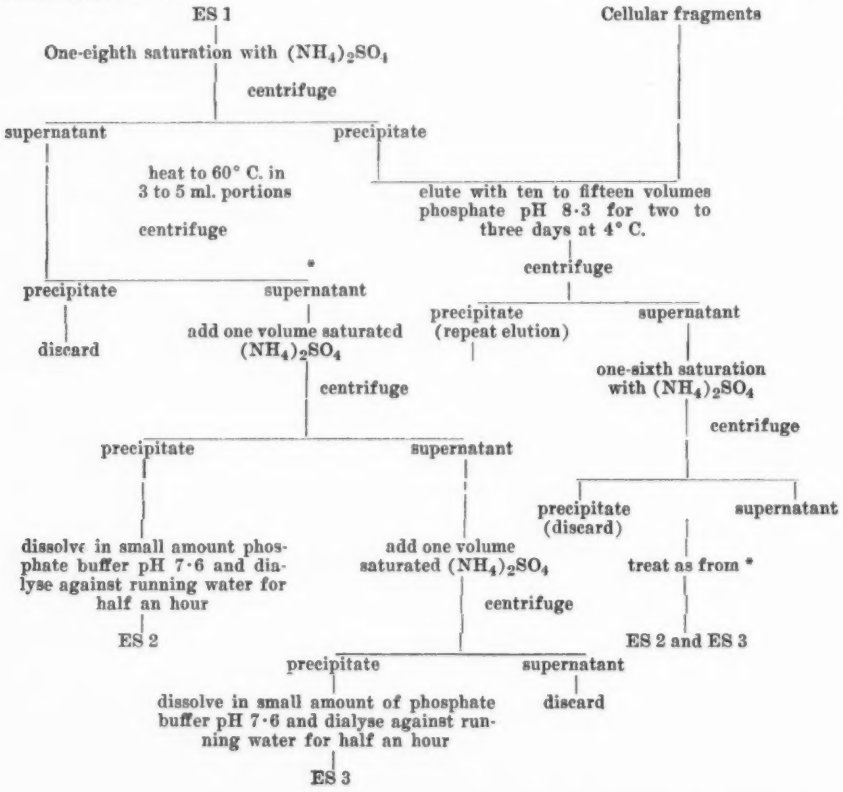
Miscellaneous Treatments.

Since none of the above treatments led to either the crystallisation of the enzyme or to its complete separation from catalase, various other methods were tried, but it has not yet proved possible to obtain an absolutely pure hydrogenase. These treatments included adsorption on substances like calcium phosphate, alumina C_γ, etc., dialysis against various molarities of sodium chloride, saturated ammonium sulphate, and precipitation into organic solvents at pHs ranging from 3 to 9.

Concentration of Hydrogenase.

On the basis of the above experiments the following method for the preparation of purified hydrogenase may be suggested:

Cells of *E. coli* are crushed three times in distilled water, conditions throughout being kept as cool as possible. The paste of bacteria and glass is diluted each time with ten volumes of glass-distilled water and the powdered glass and remaining cell particles are centrifuged down. The further purification of the pooled extracts (ES 1) and the residue is set out in the following flow sheet:



Hydrogen Acceptor Specificity.

Neither ES 2 nor ES 3 catalyses the Knallgas reaction or the reduction of nitrate. The only hydrogen acceptors are a few oxidation-reduction indicators of the type of methylene blue (MB).

TABLE 3.

The hydrogen acceptors for hydrogenase.

(The activity with MB is taken as 100; all substances were used in the amount of 0·4 ml. 2 p.c. solution per vessel.)

Substrate	E'_0 (at pH 7·0)	Activity
Methylene blue	·011	100
Nile blue	—·150	60
Thionine	·063	50
Toluylene blue	·115	50
o-cresol indo 2:6 dichlorophenol	·251	50
1-naphthalene 2-sodium sulphonate indo 2:6-dichlorophenol	·118	33
o-bromo phenol indophenol	·230	15
Benzyl viologen	—·350	13
Potassium indigo tetrathionate	—·080	10
Phenyl blue	·230	0
Methyl violet 5B	—·450	0

It is seen from Table 3 that the activity depends neither on chemical similarity nor on the E'_0. In practice only MB was used.

It was also shown that coenzyme I and cytochrome *c* are not reduced, but that in the presence of flavoprotein (prepared according to the method of Straub, 1939) plus coenzyme I a small though definite uptake of hydrogen occurs. However, neither coenzyme I nor flavoprotein affect the hydrogen uptake in the presence of MB. Riboflavin has no activity at all. These results are shown in Table 4.

TABLE 4.

Reduction of coenzyme I and flavoprotein by hydrogenase.

(Each manometer contained 0·5 ml. enzyme, and, where indicated, 0·1 ml. flavoprotein solution or 1·0 ml. coenzyme I (10 mg./ml.). The usual amounts of MB and buffer were present.)

Contents			Hydrogen uptake in µl./20 mins.
MB	CO I	FP	
+	—	—	220
+	+	—	218
+	+	+	229
—	+	—	2
—	+	+	25

Effect of the Partial Pressure of Hydrogen on the Hydrogen Uptake.

To determine the effect of the partial pressure of hydrogen on the enzyme, gas mixtures of 95, 90, 75, 50 and 25 p.c. nitrogen in hydrogen were made up and the enzyme incubated with them for various lengths of time. Atmospheres of 15 to 20 p.c. hydrogen bring about saturation of the enzyme, and this is unaffected by the initial state of oxidation of the enzyme.

The Reduction of Partially Oxidized Enzyme: The Effect of Activators.

ES 1, when incubated in Warburg flasks in an atmosphere of hydrogen for two hours, is much more active than after an incubation time of 10 minutes. An experiment was accordingly carried out to determine for how long this activation continued (Table 5).

TABLE 5.

Effect of incubating the enzyme in hydrogen.

(Contents of the cups as above; Activities in μl./15 mins.)

Time of incubation (Mins.)	3	7	30	60	120	240	720
Activity	57	76	91	108	119	184	178

It is apparent that after incubation in hydrogen for four hours the activation reaches a maximum. This is probably to be explained by the fact that in the presence of its substrate the enzyme reduces itself, a view which was expressed in a similar way by Hoberman and Rittenberg in 1943. It can easily be shown that oxygen inactivates the enzyme completely, and that the activity is wholly restored by incubation in hydrogen.

In all subsequent experiments, unless otherwise stated, the enzyme preparation used was of the type ES 3.

The possibility exists that the function of hydrogen is only to provide an anaerobic atmosphere, and that some other substance reduces the oxidised form of the enzyme. The rôle of hydrogen was proved, however, by incubating the enzyme in nitrogen *and* in hydrogen; the results are shown in Table 6.

TABLE 6.

Effect of hydrogen and nitrogen on the reduction of hydrogenase.

(Contents of the cups as above; activities in μl.)

Treatment	Hydrogen uptake in		
	10 mins.	20 mins.	30 mins.
1. Incubation for two hours in H_2	114	227	313
2. Incubation for 15 mins. in N_2, followed by admittance of H_2 up to about 15 p.c., and 4 mins.' equilibration	5	20	35
3. Incubation for 15 mins. in pure H_2, then subjected to the same manipulation as (2)	30	70	108

This experiment shows that no appreciable activation occurs in the absence of hydrogen. It might be objected that 15 p.c. hydrogen in manometer 2 was insufficient fully to saturate the enzyme, but this is disproved by the evidence provided by the rates of hydrogen uptake. In manometer 1 where there was no further activation after two hours, the reading at 20 minutes was double that at 10 minutes. In manometer 3, after 15 minutes in hydrogen, activation still proceeded fairly quickly, and the ratio of the readings (20/10 minutes) is 2·3 instead of 2·0. After 15 minutes' incubation in nitrogen, however, the ratio was 4 (manometer 2), showing extremely rapid activation, as would be expected just after the admittance of hydro-

gen. The ratio of the readings (30/10 minutes) is 3·56 instead of 3·0 in manometer 3, but 7 in manometer 2.

Besides hydrogen a number of reducing agents were found capable of activating hyrogenase (Table 7 a and b):

TABLE 7.

Effect of reducing agents on hydrogenase.

(Each cup contained 0·1 ml. of an ES 3 type enzyme preparation, 0·3 ml. of a 0·1 M reducing agent, 0·4 ml. 2 p.c. MB, and buffer pH 7·6 to 3·0 ml. Activities are in µl./10 mins.)

	Reducing agent	Incubation time	Activity
(a)	—	10 mins.	31
	Ferrous sulphate	10 mins.	200
	Thioglycollic acid (TGA)	10 mins.	146
	Glutathione (GSH)	10 mins.	151
	Yellow ammonium polysulphide	10 mins.	192
	Sodium hydrosulphite	10 mins.	204
(b)	—	120 mins.	124
	Ferrous sulphate	120 mins.	211
	Thioglycollic acid	120 mins.	173
	Glutathione	120 mins.	174
	Yellow ammonium polysulphide	120 mins.	213
	Sodium hydrosulphite	120 mins.	215

It is clear (Table 7 a) that these five reducing agents (cysteine has the same effect) could reduce the enzyme. It was not proved yet, however, that these substances and hydrogen reduced the same groups on the enzyme molecule; hence their activating effect was determined if added at once, but then incubated for two hours with the enzyme before the MB was tipped in (Table 7 b). The results show that hydrogen and the reducing agents reduce the same groups, since their effect was not additive. It may therefore be concluded that certain chemical reducing agents reduce the enzyme in the same manner as hydrogen, but in a much shorter time. It did appear probable, however, that hydrogen is necessary for the activation and that the reducing agent only acted as the catalyst, for thioglycollic acid activated much more under hydrogen than under nitrogen.

TABLE 8.

Effect of reducing agents on hydrogenase.

(The enzyme preparation, of type ES 1, had been aerated to render the possible effect of reducing agents more conspicuous. Contents of the cups as in Table 7. Activities in µl./5 mins.)

Treatment	Activity
Incubation in hydrogen for 2 hours	346
Incubation in hydrogen for 5 minutes	94
Incubation in hydrogen for 5 minutes with ferrous sulphate	96
Incubation in hydrogen for 5 minutes with TGA	114
Incubation in hydrogen for 5 minutes with cysteine	115
Incubation in hydrogen for 5 minutes with ammonium polysulphide	106
Incubation in hydrogen for 5 minutes with sodium hydrosulphite	281

The fact that thioglycollic acid, GSH and cysteine all activate the enzyme, combined with inhibitor studies (Joklik, 1950), suggest strongly that the enzyme possesses —SH groups, and that these are functional insofar as they have to be in the reduced state for the enzyme to be active.

The enzyme preparation used in the previous experiments was of the type ES 3. When ES 1 was employed, however, it was found that the reducing agents failed to activate to a significant degree (Table 8).

The only reducing agent in that group which is able to reduce this particular enzyme preparation is sodium hydrosulphite, by far the most powerful one. While both ES 1 and ES 3 type enzyme preparations are thus reduced by prolonged incubation with hydrogen, only the latter is reduced by chemical reducing agents. It appears that during the purification procedure the properties of the enzyme were altered in that respect, or that some substance was removed, which prevents the reduction of ES 1 by reducing agents.

DISCUSSION.

It has been known for a long time that hydrogenase is inactivated by oxidation; and previous authors have found that addition of metabolites such as succinate to bacteria containing oxidized hydrogenase reactivated the enzyme (Hoberman and Rittenberg, 1943). Likewise it is well known (Back *et al.*, 1946) that the enzyme is reactivated by incubation under hydrogen. However, the fact that —SH reagents are specific reactivators for hydrogenase had not been previously observed; and it is unexpected if it is assumed that the oxidative inactivation is due to the oxidation of an iron porphyrin group, i.e. the oxidation of iron to the ferric state.

The reduction of hydrogenase by hydrogen is probably due to a small amount of hydrogenase which remains in the reduced state even under fairly aerobic conditions, and which supplies the hydrogen necessary for the reduction of the –S–S– groups.

It has not yet been definitely determined whether hydrogen is necessary for the action of —SH reagents or not. The chief difficulty is that the reduction of the enzyme in the presence of reducing agent and hydrogen might be so rapid that it is impossible to say whether the reactivation is due to their combined action or to that of the activator alone. However, it appears certain that activators and hydrogen reduce the same groups in the enzyme.

Somewhat surprising is the difference in behaviour towards reducing agents by the enzyme before and after treatment with ammonium sulphate. Not only that, but it was observed throughout this work that as the preparations aged they became progressively more amenable to the influence of these chemical substances. The reasons for these phenomena are still obscure.

SUMMARY.

A method is given for the purification of hydrogenase from the extract obtained by crushing cells of *E. coli* in a ground glass mill. Certain properties of the enzyme are investigated and it is found that hydrogenase is fairly resistant to heat and organic solvents and is unaffected by dialysis or dilution. The hydrogen acceptors for the enzyme were investigated and the partial pressure of hydrogen determined at which full activity is obtained.

As expected, the enzyme is inactivated by oxidation, but it can be reactivated by hydrogen and by certain chemical substances such as cysteine or thioglycollic acid. This is taken to show the presence of labile —SH groups in the enzyme, on the integrity of which the activity depends. The significance of these results is discussed.

Acknowledgments. The author wishes to express his thanks to Professors H. Priestley and J. L. Still of the Biochemistry Department, Sydney University, for the continuous interest they have shown in this work.

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THE HYDROGENASE OF *E. COLI* IN THE CELL-FREE STATE

II. THE EFFECT OF CERTAIN INHIBITORS ON HYDROGENASE

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In recent years evidence has accumulated that hydrogenase is, or that its activity involves, an iron porphyrin protein. It has been shown that the enzyme in the oxidized state is inhibited by cyanide, azide, hydroxylamine etc. (Hoberman and Rittenberg, 1943; Lascelles and Still, 1946), and that this inhibition is removed under anaerobic conditions. In addition Waring and Werkman (1944) have demonstrated that bacteria grown on media deficient in iron contain little hydrogenase. However, each of these groups of workers used a different organism and a different technique of estimating hydrogenase activity, so that the present study was undertaken to extend the above work to *cell-free* hydrogenase.

Materials and Methods.

The preparation of cell-free hydrogenase from *Escherichia coli* strain B and its estimation have been described previously (Joklik, 1950). Cyanide was used in the unneutralized condition, since in the concentrations used the pH was found to be unaffected. Carbon monoxide was prepared by the action of concentrated sulphuric acid on formic acid, and the gas was passed through absorption towers containing alkaline sodium hydrosulphite and alkaline pyrogallol before being stored in large aspirators. Mixtures of carbon monoxide and nitrogen, and carbon monoxide and hydrogen were made up in five-litre flasks; two manometers could be gassed at the same time by displacing the gas with water. Two and a half litres of gas were found to be sufficient per manometer, and the absence of oxygen was proved by the absence of gas uptake in the presence of alkaline pyrogallol.

The light used in the experiments with carbon monoxide was provided by a series of six 200-watt globes, fixed above the surface of the bath and as close to the shaking manometer vessels as possible. A reflector was used to focus the light onto the vessels and an electric fan countered the heating effects.

EXPERIMENTAL.

Inhibitors for —SH Enzymes and General Inhibitors.

Table 1 shows the effects of those inhibitors with which inhibition was obtained. Many others, both anions and cations were tried, but were found to be without effect.

[1] This work was carried out during the tenure of a Commonwealth Junior Research Studentship during 1948.

TABLE 1.

Effect of inhibitors on hydrogenase.

Inhibitor	Concentration	P.c. inhibition
Copper sulphate	0·001 M	95
Mercuric sulphate	0·001 M	80
Stannous chloride	0·001 M	100
Silver nitrate	0·001 M	100
o-Mercuri benzoate	0·001 M	85
Phenyl mercuric acetate	0·001 M	50
o-Iodoso benzoate	0·001 M	30
Potassium sulphocyanide	0·01 M	75
Ferrous sulphate	0·033 M	75
Urethane	0·2 M	20
Urethane	1·0 M	60

Oxidizing agents such as iodine, hydrogen peroxide and potassium permanganate inactivate completely in very low concentrations, the latter two irreversibly, but the former reversibly at concentrations below 0·01 M (Table 2). The enzyme thus depends for its activity on groups which are easily oxidized, and then reduced by mild chemical reducing agents.

TABLE 2.

Effect of Iodine on Hydrogenase.

(Each manometer cup contained 0·2 ml. enzyme, 0·4 ml. 2 p.c. methylene blue (MB), the requisite amount of iodine and/or reducing agent, and buffer to make up 3·0 ml. Activities are expressed in μl./20 mins.)

Treatment	Activity
2 hours' incubation in hydrogen	207
2 hours' incubation in hydrogen + 0·001 M iodine	15
2 hours' incubation in hydrogen + 0·001 M iodine, followed by addition of 0·01 M cysteine	177
2 hours' incubation in hydrogen + 0·001 M iodine, followed by addition of 0·01 M ferrous sulphate	176

The presence of —SH groups is indicated in the following experiment in which thioglycollic acid (TGA) was used to reverse the inhibition by phenyl mercuric acetate, one of the most specific reagents for —SH groups known (Table 3).

TABLE 3.

Reversal of the inhibition of phenyl mercuric acetate with thioglycollic acid.

(Contents of the cups as above; activities in μl./20 mins.)

Contents of cups	Activity
Enzyme	81
Enzyme + TGA	146
Enzyme + phenyl mercuric acetate	58
Enzyme + phenyl mercuric acetate + TGA	128

It is of interest to note that the —SH specific inhibitors have the same effect on the intracellular enzyme as on the extracted one (Table 4).

TABLE 4.

Effect of —SH inhibitors on hydrogenase in intact E. coli.

(Each cup contained 0·2 ml. of a bacterial suspension of *E. coli*, the usual amount of MB and buffer, together with the inhibitors in a final concentration of 0·002 M. Activities are in μl./5 mins.)

Inhibitor	Activity
—	325
o-Mercuri benzoate	5
Phenyl mercuric acetate	0
Copper sulphate	0
Mercuric sulphate	0
o-Iodoso benzoate	23

Nitrite, Hydrazine, Azide and Cyanide.

None of these four reagents affected extracellular hydrogenase in the same way as the intracellular enzyme. Each of them is considered in turn:

Nitrite. Nitrite inhibits hydrogenase whether incubated with enzyme and hydrogen for five minutes or two hours. This inhibition by nitrite increases with time after the addition of MB. Table 5 shows this very clearly.

TABLE 5.

Effect of nitrite on hydrogenase.

(Contents of the cups as above. Concentration of nitrite: 0·1 M, added before gassing.)

Time after addition of MB	Per cent. inhibition for 5 mins.	After incubation 2 hours
10	10	14
20	32	30
30	54	42

It is seen that the effect of nitrite is the same whether the activity is assessed after five minutes' or after two hours' incubation with the inhibitor, and it will be noted that while the activity within the first ten minutes is scarcely affected by nitrite, that after thirty minutes is depressed by approximately 50 p.c. It appears as though a group sensitive to nitrite is exposed while the enzyme is acting on its substrate, since simply leaving the enzyme in contact with nitrite in the absence of MB failed to inhibit the enzyme.

Hydroxylamine and hydrazine. Neither of these reagents affects the cell-free enzyme markedly. Hydrazine activates if the activity is measured after a limited incubation period in hydrogen, i.e. if the enzyme is in the partly oxidized state. Hydroxylamine inhibits slightly under "aerobic" conditions, but there is no effect if it is incubated with the enzyme under hydrogen for two hours.

Azide. At pH 7·6 azide has little effect on hydrogenase: the activity is reduced by one-third at the most, the reduction of the enzyme by hydrogen is not prevented, and sometimes even an activating effect is noted (Table 6). However, if the pH is brought down to 6·0, azide inhibits almost 100 p.c. It is well-known that azide inhibits much more at an acid pH than at neutrality.

TABLE 6.

Effect of azide on hydrogenase.

Type of enzyme preparation	Time of incubation (mins.)	Concentration of azide	Activity in μl.
Aerated ES 1* at pH 7·6	5	—	21
	120	—	62
	120	M/75	44
ES 2 at pH 7·6	120	—	180
	120	M/100	254
ES 3 at pH 7·6	5	—	160
	5	M/100	149
ES 1 at pH 6·0	60	—	51
	60	M/60	2

* The meaning of the symbols has been given previously (Joklik, 1950). Briefly, ES 1 refers to the crude centrifuged extract of the crushed bacteria, ES 2 to the enzyme fraction precipitated by half saturated ammonium sulphate, and ES 3 to that precipitated by two-thirds saturated ammonium sulphate.

Cyanide. The effect of cyanide was studied more than that of any other inhibitor, since here the enzyme in the cell-free state behaved quite differently from that within the cell. Table 7 shows some of the experiments carried out.

TABLE 7.

Effect of cyanide on hydrogenase.

Experiment No.	Enzyme preparation	Incubation time	Concentration	Activity in μl.	P.c. inhibition
1	ES 1	5 mins.	—	66	
	aerated	5 mins.	0·01 M	60	9
		2 hours	—	188	
		2 hours	0·01 M	99	47
2	ES 2	10 mins.	—	57	
		10 mins.	0·01 M	86	—34
3	ES 3	5 mins.	—	21	
		2 hours	—	62	
		2 hours	0·01 M	68	
4	ES 1	5 mins.	—	64	
		5 mins.	0·0017 M	55	
		5 mins.	0·0033 M	66	
		5 mins.	0·01 M	53	
		5 mins.	0·02 M	72	
5	ES 1	2 hours	—	68	
		2 hours	0·0017 M	45	34
		2 hours	0·0033 M	40	41
		2 hours	0·01 M	34	50
		2 hours	0·02 M	26	62

Table 7 is designed to give an indication of each main type of experiment which was performed in the work on cyanide inhibition. Each experiment can be considered separately.

Experiment 1 was performed with an enzyme preparation which had just been obtained by crushing the bacteria. In addition to being subjected to strongly aerobic conditions during the preparation, it had been aerated. Nevertheless cyanide only inhibited 9 p.c., which is scarcely significant. Under prolonged incubation under hydrogen, however, the inhibition rose to 47 p.c. It can also be seen that cyanide does not combine with the oxidised form of the enzyme in such a way as to prevent its reduction, or reactivation, by incubation under hydrogen; for manometer 4 in that experiment showed a greater hydrogen uptake than manometer 1 (99 µl. as against 66 µl.). The same applies to experiment 3.

In experiment 2 cyanide activated. This behaviour was frequently shown by aged enzyme preparations.

In experiments 4 and 5 the effect of different concentrations of cyanide after short and prolonged incubation in hydrogen was investigated. Even with M/50 cyanide only 62 p.c. inhibition was obtained.

Cyanide thus inhibits more after two hours' than after two minutes' incubation; i.e. more under anaerobic than under aerobic conditions. It is unlikely that this is due to the fact that in the short term incubation experiments cyanide has insufficient time in which to act; for filling and gassing the manometers requires at least fifteen minutes, throughout which the enzyme is in contact with the cyanide, on the one hand, and on the other cyanide can activate within ten minutes, as in experiment 2.

As mentioned before, cyanide used in these experiments was unneutralized. Careful checks with neutralized cyanide showed no difference in behaviour. It was also considered that cyanide might be lost or destroyed in some way during incubation; this was disproved, however, by an experiment in which some of the contents of a manometer cup in which cyanide had been incubated with hydrogenase for two hours were used to inhibit catalase; 100 p.c. inhibition was recorded, and suitable blanks showed that this effect was indeed due to cyanide. Cyanide is thus present in an available form throughout its incubation with hydrogenase.

Carbon monoxide.

The effect of carbon monoxide was studied in gas mixtures of varying composition, after varying periods of incubation, in the presence of a number of chemical reagents, and at different pH values. The main features are shown in Table 8:

(i) The inhibition with carbon monoxide is not reversible by light, which, on the contrary, was itself found to have an adverse effect on the enzyme. Blanks carried out on yeast cytochrome oxidase showed that the lights used were strong enough to produce about 40 p.c. reversal of carbon monoxide inhibition.

(ii) The inhibition is non-competitive, since, generally speaking, the inhibition is the same at ratios of CO : H of 9 : 1 as at 1 : 3.

(iii) Carbon monoxide inhibits irrespective whether the enzyme is completely or only partially in the reduced state. In the presence of the typical iron reagent o-phenanthroline, inhibition at the lower levels of carbon monoxide is significantly decreased.

(iv) The effect is the same whether enzyme type ES 1 or ES 3 is used.

Reversal of Carbon Monoxide Inhibition and the Effect of pH.

Experiments were carried out to see whether carbon monoxide inhibition varied with the pH, and whether it could be reversed by regassing the manometers. They are summarised in

Table 9. It can be seen that the inhibition of carbon monoxide is greatest (almost 100 p.c.) at alkaline pH values, and less at 6·0 than at 7·6. It is also shown that the inhibition is over 75 p.c. reversible by sweeping out the carbon monoxide and replacing it with a hydrogen-nitrogen mixture.

TABLE 8.

Effect of carbon monoxide on hydrogenase.

(Contents of the cups as above. Activities in μl.)

Time of incubation	Reagent present	Concentration	P.c. hydrogen	Activity			P.c. inhibition
				CO	CO + Light	N_2	
10 mins.	—	—	10	50	41	136	63
			25	43	35	200	78
			50	58	51	228	74
30 mins.	—	—	10	35	34	73	52
			25	27	23	85	68
			50	41	49	137	70
2 hours	—	—	5	28	15	46	40
			10	31	26	99	68
			25	33	27	132	75
			50	84	69	128	35
			75	95	98	218	56
30 mins.	TGA	0·01 M	10	48	35	124	62
			25	59	47	183	68
			50	64	53	239	72
45 mins.	o-phenanthroline	0·01 M	10	29	28	76	62
			25	67	79	90	26
			50	83	78	109	24
10 mins.	$Na_2S_2O_4$	0·01 M	25	31	25	65	52
			60	60	50	88	32

TABLE 9.

Effect of carbon monoxide on hydrogenase.

(Contents of the cups as above, all buffers being phosphate buffers. Incubation time: 30 mins., after which the manometers to be regassed were taken off, and, after regassing with the same ration N:H gas mixture, were equilibrated ten minutes before readings were taken.)

(*a*): *Effect of pH* (Ratio throughout: 3:1).

Atmosphere	N:H	N:H	N:H	CO:H	CO:H	CO:H
pH	7·6	6·0	8·5	7·6	6·0	8·5
Activity in μl.	144	97	52	43	43	5

(*b*): *Reversibility at pH 7·6.*

Atmosphere	N:H	CO:H	CO:H	N:H	CO:H	CO:H
Ratio	3:1	3:1	3:1	2:3	2:3	2:3
Regassed with			N:H 3:1			N:H 2:3
Activity in μl.	144	43	102	234	72	183

DISCUSSION.

The view that hydrogenase is an iron porphyrin protein is based on the fact that in *intact* bacteria

(a) cyanide inhibits under aerobic, but not under anaerobic conditions; (b) carbon monoxide causes a light-reversible inhibition; (c) iron-deficiency entails low hydrogenase activity.

The inhibitor studies reported here, however, give little support to the theory that hydrogenase is an iron-porphyrin enzyme as such. If it is, the properties of the iron-porphyrin group towards inhibitors are masked in hydrogenase in the cell-free state.

The most vulnerable groups of hydrogenase, both inside and outside the cell, are undoubtedly the —SH groups, since very strong inhibition was obtained with substances like o-mercuribenzoate, phenyl mercuric acetate, o-iodosobenzoate, iodine, and copper and mercury ions. In the case of iodine and phenyl mercuric acetate almost complete inhibition could be *reversed* by the addition of a compound containing —SH groups, e.g. cysteine and thioglycollic acid.

Hydroxylamine and hydrazine failed to inhibit the enzyme significantly. In particular, the strong inhibition caused by these compounds under aerobic conditions in the intact cells, reported by both Hoberman and Rittenberg for *Proteus vulgaris* (1943), and by Lascelles and Still (1946) for *E. coli*, was entirely absent.

The maximum inhibition observed with cyanide was about 50 p.c., not, as would be expected, in the oxidized state, but after two hours' incubation under hydrogen. Cyanide completely failed to prevent the reduction of the enzyme from the state where only 20 p.c. of the maximum activity is displayed to the fully reduced condition. It is unlikely, therefore, that cyanide combines with the oxidized prosthetic group.

The inhibition caused by carbon monoxide was largely independent of the carbon monoxide : hydrogen ratio; it was rarely over 70 p.c. even at ratios of 19 : 1. The inhibition was not reversible by light, and completely unaffected by previous incubation in hydrogen or the presence of reducing agents. It was greatest at alkaline pH values, and largely reversible on the removal of carbon monoxide.

The marked contrast in the properties of the enzyme in the intact cell and in the extra-cellular state (absence of that inhibition with cyanide, azide, hydrazine, etc., which was reported for the intact cell by Hoberman and Rittenberg, 1943; Lascelles and Still, 1946, and Farkas and Fischer, 1947) seems to indicate that during extraction the properties of an enzyme system can be changed to a profound degree, and emphasizes the fact that great caution is necessary in extending inhibitor studies on intracellular enzymes to systems in extracts or homogenates.

The most recent work seems to indicate that the reduction of MB in the

presence of hydrogen involves not only the enzyme activating hydrogen, but also a carrier or perhaps even another enzyme system. It might prove possible to separate the enzyme responsible for the exchange reaction (Hoberman and Rittenberg, 1943) from the complex causing the reduction of MB. This would explain the results of Hoberman and Rittenberg (1943), who were able to inhibit MB reduction while the exchange reaction between hydrogen and water was unaffected; they were also able to induce the reduction of MB in organisms which could only catalyse the exchange reaction by the addition of fumarate.

The actual hydrogen acceptor for hydrogenase, however, is at present unknown. Neither coenzyme I nor cytochrome c are reduced and flavoprotein is reduced only very slightly. An indication of the natural hydrogen acceptor, if such a one exists—that is, if the function of hydrogenase is not primarily to work in reverse—will be very important in assessing the physiological significance of the enzyme.

SUMMARY.

A cell-free preparation from *E. coli* has been used to study the effect of inhibitors on the enzyme hydrogenase.

Inhibitors known to inhibit —SH enzymes inhibit hydrogenase. This inhibition is completely reversible in the case of dilute iodine and phenyl mercuric acetate on the addition of compounds containing —SH groups, such as thioglycollic acid and cysteine.

Nitrite causes a rapidly increasing inhibition as the enzyme acts on its substrate. It is suggested that groups essential for hydrogenase activity, and only exposed while the enzyme is active, are attacked by nitrite.

Cyanide does not inhibit the enzyme to any large extent. It does not prevent the reduction of the enzyme, showing that the oxidized functional group of the enzyme cannot combine with cyanide. Up to two hours, the inhibition increases with the length of the incubation period in hydrogen.

The inhibition caused by carbon monoxide is unaffected by previous incubation in hydrogen and by the presence of —SH reducing agents. The inhibition is largely reversible by replacing the carbon monoxide-hydrogen with a nitrogen-hydrogen mixture, and is greater at alkaline pH values than at acid ones.

These results are discussed in the light of present day knowledge of hydrogenase.

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THE DEMONSTRATION OF COMPLEMENT-FIXING ANTIBODIES TO HERPES FEBRILIS VIRUS

by MALCOLM FOWLER

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In recent years complement fixation tests have been used with increasing frequency in the diagnosis of virus infections, largely because the ease with which viruses may be propagated in chick embryos has facilitated the preparation of suitable antigens. Up to the present time, however, little success has attended attempts to demonstrate complement fixation in infections due to the virus of herpes febrilis. Positive results have been elicited with difficulty using antigens derived from infected animal tissues and not at all with those produced by means of the developing egg.

Todorovitch (1925), using herpetic vesicle fluid and serum from an immunized monkey, and Stroian (1929), using serum and tissue extracts of immune rabbits against a herpes antigen, each noted complement-fixation. With an antigen prepared from the infected guinea-pig, Bedson and Bland (1929) succeeded in demonstrating complement fixation to a low titre. They prepared their antisera in guinea-pigs by 6 or 8 intraperitoneal inoculations of 1 or 2 ml. of a 10 p.c. tissue suspension containing virus. Brain (1932), using their technique, obtained a positive reaction with the sera of 30 out of 32 individuals with a history of herpetic infection. Myers and Chapman (1937) using immunized rabbits as a source of antiserum tested many antigens, including the chorio-allantoic membrane. With a 10 p.c. emulsion of rabbits' testis as the antigen their sera showed titres up to a dilution of 1/64. This result, however, they did not regard as significant apparently because of the uncertain behaviour of their controls.

Dr. F. M. Burnet suggested to me that an attempt should be made to devise a complement fixation test, using as likely antigens the chick embryo liver (infected by the intravenous route) and the chorio-allantoic membrane. No fixation was demonstrable either with rabbit antisera, which were almost invariably anticomplementary, or with sera from herpetic individuals.

The following technique, founded on a different approach, has produced successful results. The basis for the preparation of the antigen is the discovery by Rose and Molloy (1947) that after yolk-sac inoculation herpes virus can be recovered from the allantoic fluid in high titre.

Methods.

Preparation of the antigen. The HFE egg-adapted strain of herpes virus obtained from the Walter and Eliza Hall Institute of Medical Research by the courtesy of Dr. Burnet, was used throughout. The frozen-dried material was passaged several times on the chorio-allantoic membrane. The initial material for yolk-sac inoculation consisted of 4 such membranes ground in 5 ml. of sterile nutrient broth. It was injected in amounts of 0·25 ml. into 7-day chick embryos. On the second day after incubation at 35° C. the embryos were usually moribund, infected embryos looking haemorrhagic and under developed. The allantoic fluid was harvested and titrated on the chorio-allantoic membrane, fluids with titres below 10^{-5} being discarded. As Nagler (1946) found a high titre of virus in the amniotic fluid following yolk-sac inoculation with herpes, accidental rupture of the amniotic sac was disregarded. For further yolk-sac passage infected allantoic fluid was used. Allantoic fluid from normal 9-day eggs constituted the control.

Following light centrifugation to bring down coarse particles, test and control fluids were each centrifuged for 2½ hours at a speed of 5,000 revolutions per minute, the sediment being resuspended in one-fifth of its original volume of calcium magnesium saline. The supernatant fluid was also kept for testing. The manoeuvre of centrifuging was suggested by Bedson's experience (1927) with herpes virus derived from infected guinea-pig tissue.

Preparation of the antiserum. The success of Bedson and Bland in immunizing guinea-pigs with herpetic material by the intraperitoneal route initiated the following procedure.

Guinea-pigs were given weekly intraperitoneal injections of emulsified mouse brains, infected with herpes febrilis by the intracerebral route. Each infected brain was suspended in 1·5 ml. of sterile 50 p.c. glycerol saline and kept for a few days at a temperature of 4° C. The brains were ground to form a suspension in the same glycerol saline. The initial dose was 0·5 ml. followed by one of 1 ml. and six doses of 2 ml. A control series of guinea-pigs was treated in a similar manner with normal mouse brains. The sera of both groups were tested for neutralizing antibodies by titration on the chorio-allantoic membrane, according to the method of Burnet and Lush (1939). Specific antibodies were only detected with the test sera, when anti-pock activity was marked.

Sheep's red blood cell-haemolysin system. A 6 p.c. suspension of washed and packed sheep's red blood cells was sensitized with an equal volume of 6 M.H.D. of appropriate haemolysin.

Complement. In the test proper 2 minimal haemolytic doses were used. The diluent throughout was calcium magnesium saline.

Complement titration. Although Bedson and Bland (1929) found that heating rendered fresh guinea-pig sera anticomplementary, no such effect manifested itself here. Before centrifugation and resuspension the antigens were sometimes procomplementary, but after such treatment this annoyance was eliminated.

Test Proper.

In order to save reagents the drop technique was employed throughout. Each drop (0·06 ml.) comprised one volume. The components were mixed in the usual manner and incubated in a water bath for one hour at 37°C. After the addition of the fourth volume, that is, the indicator system, the test was read at the end of a further half-hour's incubation.

Controls. The test and control sera were each tested for combined fixation with both test and control antigens. Each reagent was also tested singly. In addition, antigens were tested against the sera of several normal guinea-pigs from the stock cages. Finally, the test antiserum was set up against an emulsion of chorio-allantoic membrane infected with vaccinia.

All reagents were tested for bacteriological sterility.

Results.

The results, which are consistently reproducible, are embodied in Table 1.

TABLE 1.

Complement fixation using 2 M.H.D. of complement.

Antigens	Sera Test ½	¼	⅛	1/16	1/32	1/64	1/128	1/256
Sediment								
Test	++++	++++	++++	++++	++++	++++	++	—
Control	—	—	—	—	—	—	—	—
Supernatant								
Test	++++	++++	++++	++	+	—	—	—
Control	—	—	—	—	—	—	—	—

Antigens	Control ½	¼	⅛	1/16	1/32	1/64	1/128	1/256
Sediment								
Test	—	—	—	—	—	—	—	—
Control	—	—	—	—	—	—	—	—
Supernatant								
Test	—	—	—	—	—	—	—	—
Control	—	—	—	—	—	—	—	—

++++ = complete fixation. — = no fixation. Other controls all negative.

DISCUSSION.

The success of the above technique would seem to lie in the method of preparation of the antigen, which results in the production of large quantities of virus in clear or opalescent allantoic fluid of a low protein content, which minimizes non-specific fixation.

An interesting feature is the relatively high titre obtained with the supernatant fluid. To determine whether this was due to a "soluble" antigen the raw allantoic fluid was filtered through a collodion membrane of 100 $\mu\mu$ pore size. The filtrate did not fix any complement in the presence of specific antisera. The sediment must therefore contain either particles of aggregate virus, small virus particles being left in the supernatant fluid or, more likely, virus adsorbed on fragments of non-specific organic matter.

The possible use of the complement fixation reaction as a diagnostic procedure for the detection of infected human beings should be kept in mind; this aspect is now being investigated.

The test may prove to be of considerable value in the study of experimental infection and immunization with the virus of herpes febrilis and in the elucidation of problems connected with the natural disease and with virus structure.

SUMMARY.

Complement fixation has been demonstrated against the virus of herpes febrilis, using as antigen allantoic fluid of eggs infected by the yolk-sac route. Guinea-pigs immunized by intraperitoneal inoculation furnished the specific antibody.

Further developments which may follow the application of the test, as a routine laboratory procedure and in fundamental research, are briefly indicated.

Acknowledgments. My thanks are due to Dr. F. M. Burnet for his encouragement and advice during my stay at the Walter and Eliza Hall Institute of Medical Research, and to Dr. Charles Swan for his help in the preparation of the manuscript.

ADDENDUM.

Since this investigation was submitted for publication a paper by M. E. Hayward entitled "Serological Studies with Herpes Simplex Virus" (British J. exp. Path., Vol. XXX, p. 520, 1949) has been received (9/5/50). Complement fixation is described using various antigens including allantoic fluid similarly prepared. This author found that the agent mainly responsible for fixation was contained in the supernatant fluid and that the sediment, except in the case of infected chorio-allantoic membranes, showed no complement fixing activity. It was considered that the "soluble" antigen was difficult to remove from tissue particles spun down with the virus in infected chorio-allantoic membranes. This same concentration may have occurred in the sediment from fluids containing much yolk-sac material, as recorded in the present paper. On filtration adsorption of the small particle on to the collodion membrane may have taken place.

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THE ACTION OF TROPINE AND HELIOTRIDINE-ALKALOIDS ON EXCITATION, PROPAGATION AND RECOVERY IN MUSCLE

by E. M. TRAUTNER AND I. A. N. McCALLUM[1]

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(Accepted for publication 18th November, 1949.)

The present work is a continuation of the work of MacCallum, McCallum and Shaw, and of Shaw, Agar and McCallum on the action of yohimbine on the nerve and muscle of the toad. Esterified alkaloids of the tropine and heliotridine group were investigated for action on the diphasic electrical response of the sartorius of the toad *Bufo marinus*. The effects of the alkaloids and the chemical groupings responsible for the different effects are characterized; analysis and interpretation of the effects will be attempted in a following publication.

MATERIAL AND METHODS.

The sartorius preparation was made with the usual precautions to avoid skin contamination and damage to the muscle fibres. The excised muscle was mounted in a paraffin chamber in frog Ringer solution of room temperature, 18–22° C., and allowed to rest for at least 30 minutes. Preparations which within 45 minutes did not give normal reproducible responses were discarded.

When the preparation had proved to be satisfactory the bath was emptied and refilled with a solution of the alkaloid in frog Ringer. Alkaloid concentration was chosen so that the toxic effect appeared slowly during 15–30 minutes, remained steadily progressive and could be followed for 1–2 hours. Dependent on the alkaloid, concentrations between 1:1,500 and 1:4,000 were found to be optimal for the purpose.

The bath was emptied immediately before and refilled immediately after stimulation; prolonged contact of the muscle with air was avoided. As untreated muscles gave constant responses for three to four hours it can be accepted that the manipulations did not affect the results.

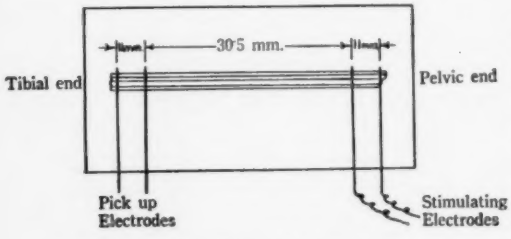


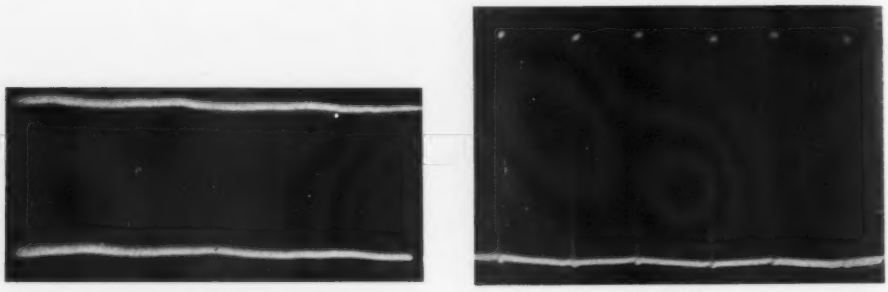
Fig. 1. Experimental arrangement.

Stimulation was effected at the nerve-free pelvic end of the muscle by two silver wire electrodes transversely applied; the response was collected by two silver wires 1 cm. apart transversely applied at the tibial end of the muscle. The response was recorded through amplifiers directly coupled to an oscillos-

[1] This work was aided by grants from the National Health and Medical Research Council, Canberra.

cope; detailed description of the apparatus is given by Shaw and co-workers (1949). Fig. 1 shows the arrangement of the bath.

All alkaloids examined raise the threshold of stimulation. The intensity of the stimulation was therefore chosen high enough above the threshold of the untreated muscle to assure that it could be maintained at equal strength throughout the experiment. The threshold of the untreated muscles varied from 0·3 to 1·5 volts; the stimulation used was between 2 and 4·5 volts, with an average of 3·5 volts and 1 millisecond duration.



(1). (2).

Fig. 2. Scale of oscilloscope recordings: (1) Potential: vertical distance: 10 millivolt. (2) Time of conduction: distance between dots: 10 milliseconds.

The action potential was measured as the vertical distance between zero level and upper spike of the diphasic response on the oscilloscope, the time of conduction as the horizontal distance between stimulation and onset of the visible response. Fig. 2 gives the time and voltage scale as projected on the oscilloscope. In many experiments the tension was measured by a strain gauge which allowed only minimal shortening of the muscle.

The action potential and time of conduction of the untreated muscle, referred to as the original values, differ widely in magnitude as well as in proportion. Fig. 3 shows a series of findings, the action potential being plotted against the time of conduction in the proportions as recorded on the oscilloscope with the stimulus used for the experiment. No evidence was found to indicate that the varying magnitudes of the original values affect the action of the alkaloids to any noticeable degree.

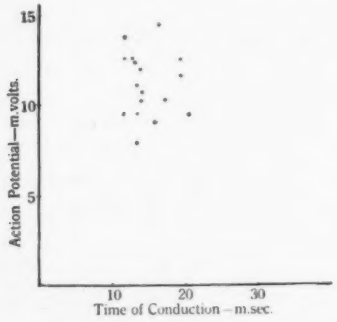


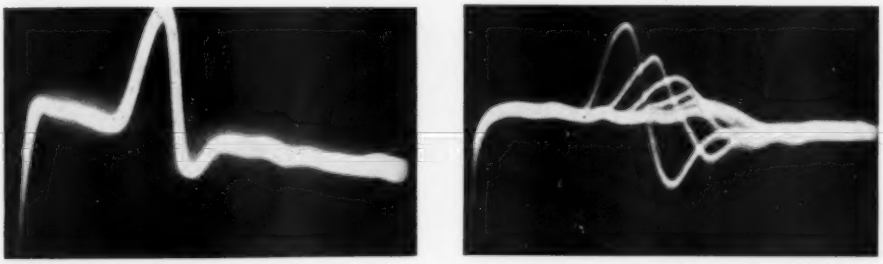
Fig. 3. Variation of Action Potential and Time of conduction in untreated muscles.

RESULTS.

General Remarks.

Some alkaloids were available only in small amounts as hydrobromides; sodium hydrobromide was tested in identical and slightly stronger concentrations and found to have no effect on the preparation.

To ascertain whether the toxic effect of the alkaloids was due to the basic component alone or to the acid alone, tropic acid and tropine were tested singly as well as in mixtures of equimolecular proportions; none of these showed any activity. Fig. 4 shows a repetitive response at the rate of 5 stimuli per second to a muscle under the influence of equimolecular proportions of tropic acid and tropine for 60 minutes and of an identical concentration of atropine for 50 minutes.



(1). (2).

Fig. 4. (1) Equimolecular mixture of tropine and tropic acid corresponding to 1:2,000; repetitive stimulation, 5 per second after 60 minutes. (2) Atropine 1:2,000; repetitive stimulation 5 per second after 50 minutes.

All esterified alkaloids examined lead finally to abolition of the response, action potential as well as contraction. In the last stages before abolition the diphasic responses as recorded by the oscilloscope are so flat and rounded that neither the onset of the wave nor the spike of the action potential can be ascertained with any accuracy. These last stages have not been considered in the present investigation; the effect of the alkaloid was followed until the action potential dropped to about one-fourth of its original value. It is assumed that within this interval the recordings are reliable and comparable.

The time in which with identical concentrations complete abolition of the response takes place may be taken as a measure of the toxicity of the alkaloids to the muscle. It was found that these times are of about the same order for all the alkaloids examined. Of the tropine bases only those esterified with tropic acid show mydriatic action and of the heliotridine bases lasiocarpine only has a pronounced physiological action (causing haemorrhages, mainly in the liver); it will therefore appear that the effect on the isolated muscle is not connected with the typical pharmacological action.

In their effect on the isolated sartorius the alkaloids examined fall into two sharply distinguished groups, one in which the responses obtained on repetitive stimulation are not altered, and one in which they are affected in a surprising degree. The former effect will be called the "depressant effect", the latter the "delayed-recovery effect."

The Depressant Effect.

The following alkaloids lead to a weakening and final abolition of responses without any effect on the responses obtained on repetitive stimulation: valeroidine, poroidine, iso-poroidine, valeryl-tropine and iso-heliotrine. The first four are tropine bases and by-alkaloids of *Duboisia myoporoides*, the latter is a heliotridine base and has been extracted from *Heliotropium Europeum*. The chemical configuration which distinguishes these alkaloids from those showing the delayed-recovery effect will be discussed later.

Fig. 5 gives a series of responses obtained with valeroidine. At suitable concentrations almost identical records are obtained with the other alkaloids mentioned; it was therefore not considered necessary to deal singly with the individual alkaloids. The changes observed can be detailed as follows:

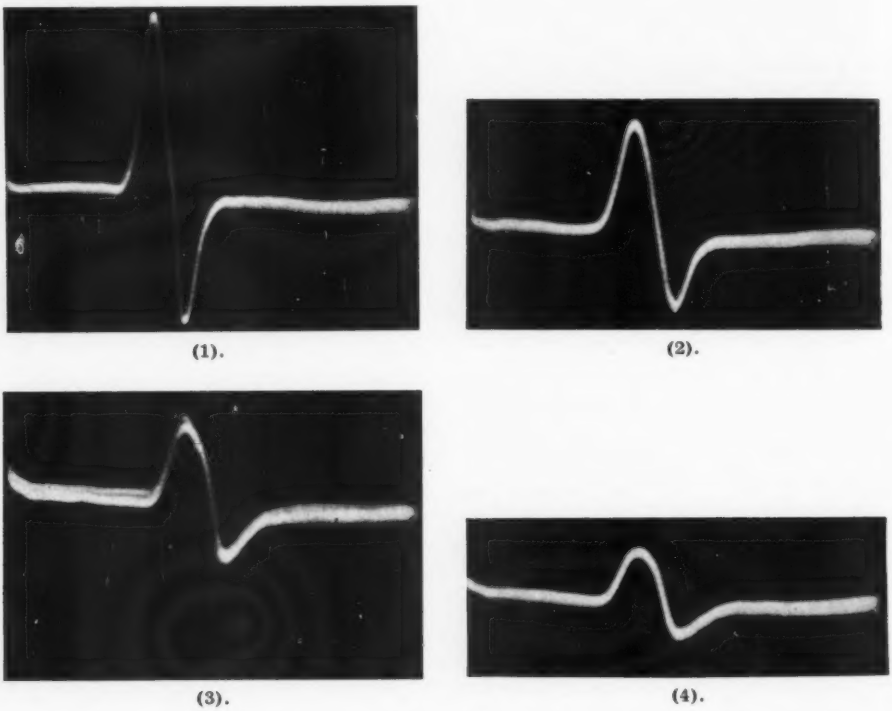


Fig. 5. Depressant effect: Valeroidine 1:2,000; 2·2 v.—1 m.sec.; 20·5°. (1) Response of untreated muscle. (2) Single response after 45 minutes. (3) Repetitive stimulation 5 per second after 60 minutes. (4) Repetitive stimulation 5 per second after 90 minutes.

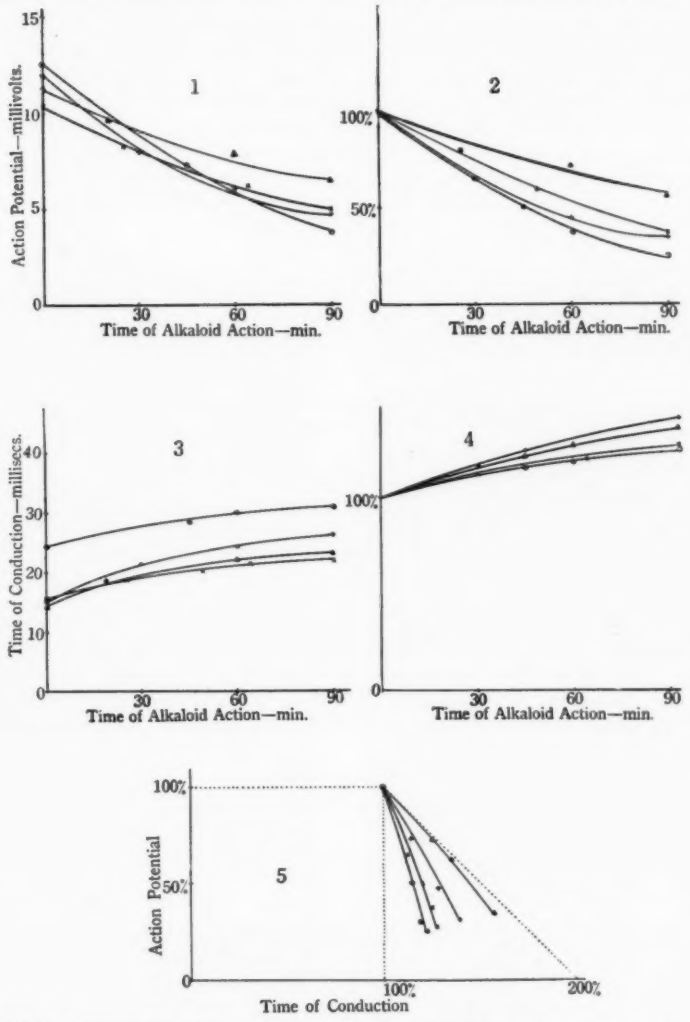


Fig. 6. Depressant effect. Changes of action potential and time of conduction. (1) Decrease of action potential in millivolt with the time of alkaloid action. (2) Decrease of action potential in per cent. taking the original value as 100 p.c. (3) Increase in time of conduction in milliseconds with the time of alkaloid action. (4) Same in per cent. taking the original time of conduction as 100 p.c.

Relative changes of action potential and time of conduction. (5) Action potentials plotted against the corresponding times of conduction in per cent. of the original value.

O Valeroidine 1:2,000; 2·2 v.—1 m. sec.; 20·5°.
\+ Base 21:2,000; 3 v.—1 m. sec.; 22°.
△ Valeryl tropine 1:2,000; 4·5 v.—1 m. sec. 19·5°.
× Heliotrine 1:2,000; 3 v.—1 m. sec. 21°.

Action potential. The action potential drops progressively with the time of alkaloid action. Fig. 6 (1) shows the changes observed in several experiments. A better comparison of the results of different experiments is achieved if the observation data are not plotted in actual size but in per cent.-changes taking the original value (that of the untreated muscle) as a common unit; this method of drawing the curves is accepted for the following graphs, the original value being taken as equal to 100. Fig. 6 (3) gives the same results as 6 (1) in per cent.-changes. It is seen that the action potential decreases, more quickly at the start of the experiment and more slowly later on, in a smooth curve until the responses become either constant or abolished; there is no sharp angle suggesting a discontinuity of the curve. The results from different alkaloids are so similar that it would appear that with suitable concentrations identical curves could be obtained. Actually this is not the case; the curves are not predictable, they may be slightly rounder or flatter than expected, although they vary only within a narrow margin. All that can be said is: that the curves are characteristic and similar enough to be considered to conform to one pattern.

Apart from a possible quicker action in producing the depressant effect no difference could be found between the secondary bases poroidine and iso-poroidine (present in *Duboisia myoporoides* as an inseparable mixture, Base Z) and the other alkaloids which are all tertiary bases.

Time of conduction. The time of conduction increases steadily up to 50 p.c. and slightly more over its original value. Fig. 6 (3) shows the actual data, Fig. 6 (4) the per cent. changes with time of alkaloid action for the same experiments as 6 (1) and (2). On superficial inspection it may appear that the curves are of a similar pattern to those obtained for the action potential only marking increases instead of decreases; some are so similar that they may almost be proportional: they start with a smooth curvature, the radius of curvature increasing steadily as if the time of conduction approaches a maximum value. Others, however, increase in an almost linear function. As with the action potential, the results vary with the muscle and the drug and are not exactly repeatable although the variation keeps within a fairly narrow margin.

Relative changes of action potential and time of conduction. If the values of action potential and time of conduction of the individual responses, measured in per cent. changes with a common value for the original response are plotted against each other, the relative changes are obtained. If the changes of both values were equal in relation to the original values, a line descending at 45° inclination would be obtained under the condition of the graph; it is seen that in the depressant effect the increase in the time of conduction *lags* relatively to the decrease in action potential; the resultants are sometimes almost rectilinear, more often they are curves with a big radius of curvature whose centre is on the same side as the crossing of the axes; they revolve clockwise. The actual inclination of the resultants is, of course, dependent on the scale in which action potential and time of conduction are plotted; the direction of curvature, however, is unaffected by linear variation of the scale.

Tension. The tension developed on contraction appears to drop parallel with the action potential and is abolished at the same time; no relation to the time of conduction could be observed.

General shape of the diphasic response. The characteristic angular shape of the normal diphasic response is maintained until the action potential drops to about a quarter of its original value; the wave leaves the horizontal level fairly abruptly, both the upper and the lower spike of the response remain sharp and the return to zero level is only slightly more smooth than in the original response, suggesting that the factors which condition the shape of the normal response remain active and correlated for a long time. Only in the last stages of the experiment, at the time near abolition of the response, does the wave lose its angularity and become flat and rounded.

Repetitive stimulation. On repetitive stimulation at the rate of 5–7 stimuli per second identical responses are obtained. The records on the oscilloscope are exactly superimposed in time of conduction as well as action potential and shape and duration of the wave. Only at the very latest stages near abolition blurred and no longer covering responses are obtained.

Duration of the wave. The duration of the response as measured from the onset of the wave to return to zero level remained in most experiments almost unchanged throughout the period of observation; it does certainly not alter parallel with the action potential. Whether it is to a noticeable extent affected by the change in time of conduction is difficult to ascertain. In some experiments a lengthening was observed, but not in others. After the action poten-

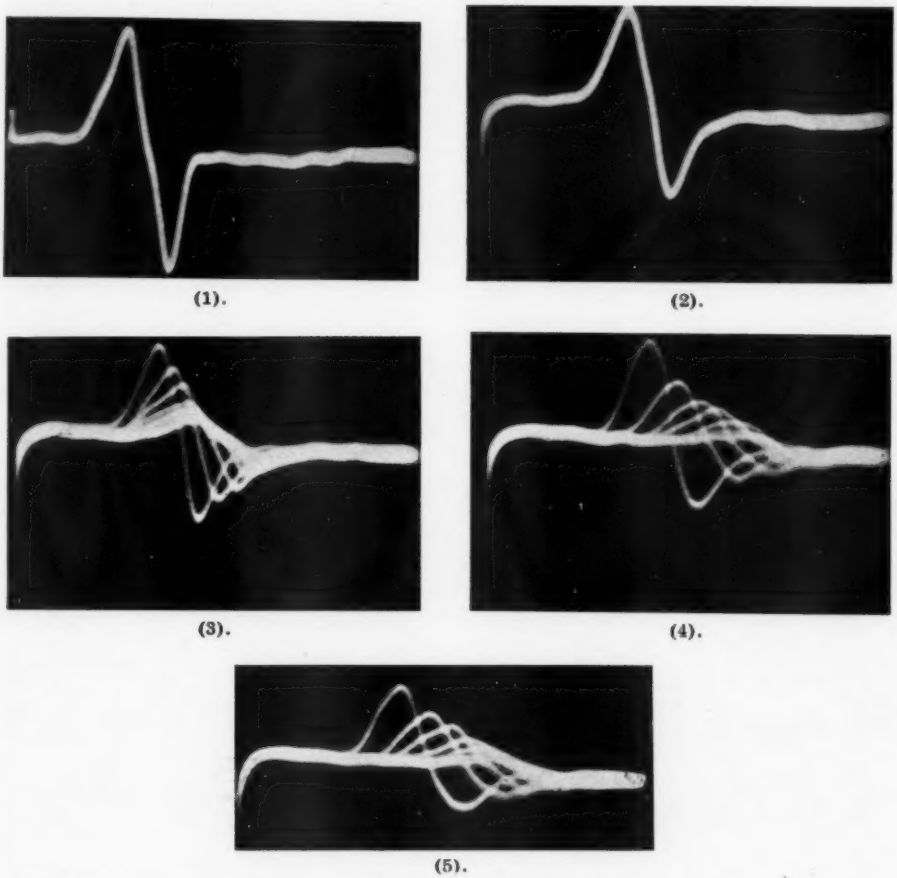


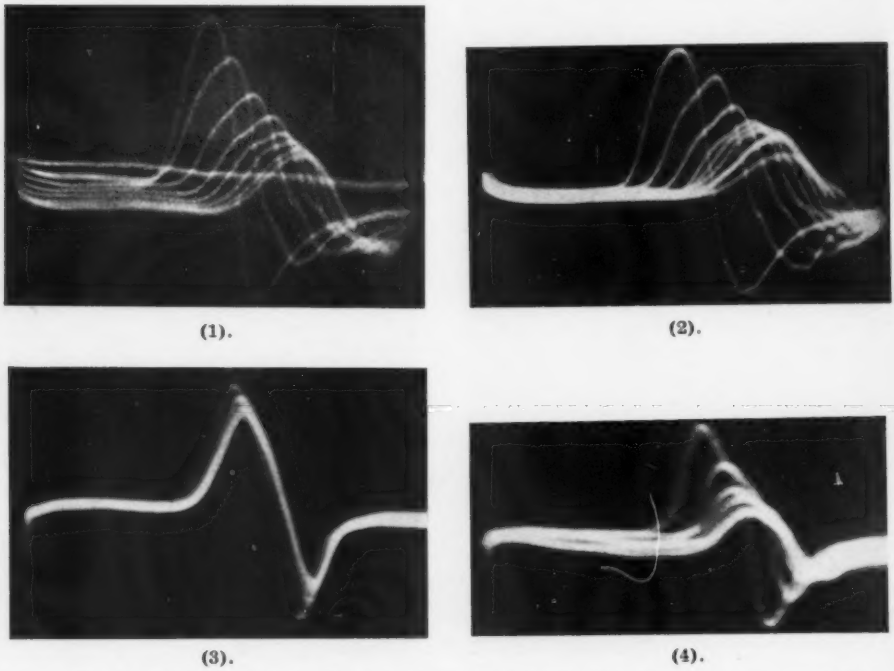
Fig. 7. Delayed-recovery effect. Atropine 1:2,000; 2·2 volts—1 m. sec. 19·5°. (1) Response of the untreated muscle. (2) Single response after 15 minutes. (3) Repetitive stimulation at the rate of 5 stimuli per second after 15 minutes. (4) Repetitive stimulation at the same rate after 70 minutes. (5) A second repetitive stimulation about 5 seconds after the previous one (4).

tial has dropped to about a quarter of its original value the wave is too rounded to allow precise measurements of its duration.

The threshold increases slightly but variably during the time of alkaloid action, the average increase being less than 1 volt.

The Delayed-Recovery Effect.

This effect has been observed with atropine, apo-atropine, hyoscine, tigloidine (a by-alkaloid of *Duboisia myoporoides*) and lasiocarpine (in *Heliotropium Europeum*). All these alkaloids lead to a weakening and final abolition of the response similar to the effect previously described; but *in addition* to it they show a quick, though short-lived weakening of the responses on repetitive stimulation. The effect is referred to as "fully developed" if the action potential drops within three to four stimulations at the rate of 5 per second to about one-half of the value it had in the first response. If possible, the alkaloid concentrations were chosen so as to produce the fully developed effect within 15–20



(1). (2). (3). (4).

Fig. 8. Delayed-recovery effect. Lasiocarpine 1:2,000; stimulus 3 v.—1 m. sec.; 5 per sec. 20°. (1) After 30 minutes of alkaloid action. (2) A second repetitive response about five seconds after the first, 45 minutes of alkaloid action.

Hyoscine 1:2,000; 3 v.—1 m. sec.; 5 per sec. 19·5°. (3) After 15 minutes; effect not fully developed. (4) Fully developed effect after 60 minutes.

minutes; under these conditions a period of 1 to 1½ hours was available for observation of the effect before the responses were too weak or abolished.

Fig. 7 shows a series of responses obtained with atropine 1:2,000; Fig. 8 two each obtained with hyoscine and lasiocarpine 1:2,000. If the effect is fully developed the records from different alkaloids are very similar; it is not possible more than to guess from which alkaloid a record has been obtained. The essential changes are almost identical to those described by Shaw and co-workers (1949) in the action of yohimbine on the nerve and muscle of the toad; they can be detailed as follows:

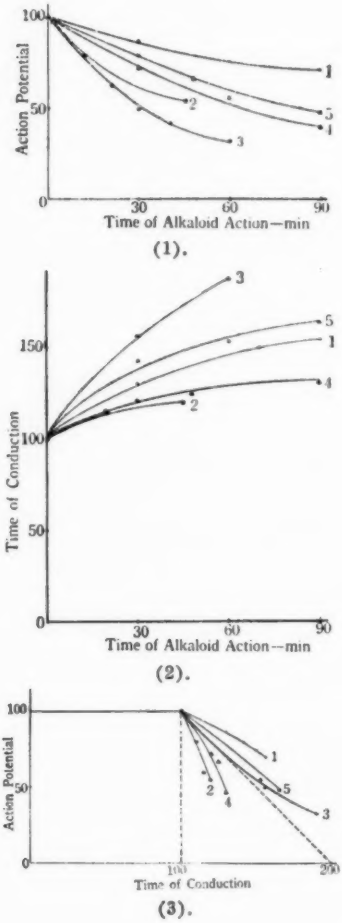


Fig. 9. Isolated responses with alkaloids causing delayed recovery.

(1) • Atropine 1:2,000; 19·5°; 3·1 v.—1 m. sec.

(2) O Atropine 1:1,500; 20°; 4·4 v. —1 m. sec.

(3) + Tigloidine 1:1,500; 21°; 3·5 v.—1 m. sec.

(4) △ Lasiocarpine 1:2,000; 20°; 3 v.—1 m. sec.

(5) × Hyoscine 1:2,000; 19·5°; 3 v. —1 m. sec.

Graph 1. Decrease of action potential with time of alkaloid action taking the original value as 100. Graph 2. Increase in time of conduction with time of alkaloid action taking the original value as 100. Graph 3. Relative changes of action potential and time of conduction, obtained by plotting the corresponding values from graph 1 and 2 against each other.

Isolated, Single Responses.

If the muscle is stimulated at well-spaced intervals, say every ten minutes, during the time of action of a delayed-recovery drug the responses obtained are similar to those described under the depressant effect. Fig. 9, (1) and (2) show for a series of experiments the changes of action potential and time of conduction in per cent. of the original values plotted against the time of alkaloid action. The action potential drops in a smooth curve of about the same pattern and a similar range of variation as obtained in the depressant effect; the curves representing the changes in time of conduction are, however, more widely scattered and the per cent.-increase is generally higher. In the record of tigloidine used for the graph the action potential decreases in 90 minutes about 25 p.c. and the time of conduction increased 50 p.c. Fig. 9 (3) shows the relative changes of action potential and time of conduction plotted as the corresponding curve for the depressant effect. The curves are on both sides of the 45° line, some are almost rectilinear, others have a clockwise direction of curvature as observed for the depressant effect, but the experiment with tigloidine shows an anti-clockwise curvature. The relation of the changes appears to be looser than in the depressant effect; in some experiments the relative increase of the time of conduction is smaller than the decrease of action-potential, in others it is equal or even exceeds it. The threshold rises slightly in about the same order as in the depressant effect.

The most obvious difference in isolated responses of the two kinds of drugs is seen in a *slower, more rounded return from the lower spike of the diphasic response to zero level*; the return is delayed and the delay is marked enough to distinguish isolated responses of the fully developed delayed-recovery effect from those of the depressant effect. This characteristic delay becomes more pronounced as the action of the alkaloid proceeds, finally rounding and stretching the second half of the diphasic response to an extent which makes it impossible to ascertain the exact position of the return to zero level. The potential difference disappears so slowly that even apparent zero cannot be taken as proof that there is not between the pick-up electrodes still a potential difference too small to be recorded by the oscilloscope and that the system has really recovered to the condition in which it was before stimulation. That it has not done so is shown if a second stimulus is applied immediately after the first.

EXPLANATION OF FIGS. 10 AND 11.

Fig. 10. Delayed-recovery effect. Atropine 1:2,000; 19·5°, stimulations at the rate of 5 per second, 3 v.—1 m. sec. after

• = 15 minutes. △ = 70 minutes.
O = 30 minutes. × = 90 minutes.
\+ = 50 minutes.

Graphs 1 and 2. Changes of action potential and time of conduction taking the values of the untreated muscle as unit (100 p.c.). Graph 3. Relative changes obtained by plotting the corresponding values of action potential and time of conduction from graphs 1 and 2, against each other.

Fig. 11. Delayed-recovery effect. Graphs 1 and 2. Action potential (graph 1) and time of conduction (graph 2) in repetitive responses, 5 per second, compared with the original value of the untreated muscle as unit (100 p.c.) and marked with a circle on the ordinate. Graph 3. Relative changes of action potential and time of conduction obtained by combining the corresponding values from graphs 1 and 2.

Hyoscine (continuous line) 19·5°; 1:2,000, 3 v.—1 m. sec. + after 30 minutes. • after 60 minutes.

Tigloidine 1:1,500 (interrupted line) 3·5 v.—1 m. sec. 21°. O after 35 minutes. • after 60 minutes.

Lasiocarpine 1:1,500 (dotted line) 3 v.—1 m. sec. 20°. × after 45 minutes, third repetitive stimulation after two previous ones with about 5 seconds interval. △ after 90 minutes.

It has not been considered necessary to reproduce a series of isolated single responses throughout the effect of a delayed-recovery drug because the *first* response obtained in a repetitive burst represents such a single response.

Repetitive Stimulation—Delayed-Recovery Effect.

This effect is observed if a second stimulation is applied to the muscle at a very short interval after the first. The effect develops gradually; after 10 to 15 minutes of alkaloid

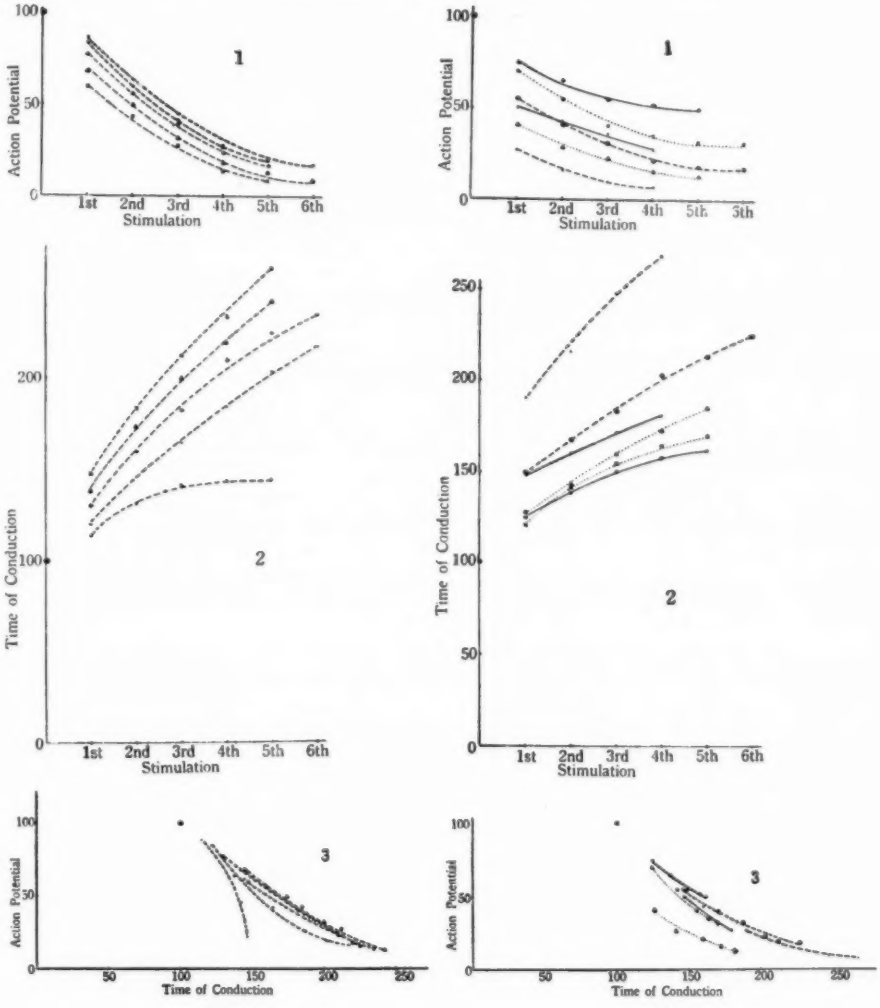


Fig. 10.

Fig. 11.

action it can just be noticed on rapid repetitive stimulation that the action potential drops a little and that the time of conduction is slightly increased (Fig. 11 (4)); later on the effect is very pronounced even if the stimulations are placed at an interval of one second or longer. The characteristic changes are as follows:

Action potential. Fig. 10 (1) shows the decreases of action potential with respect to the original value obtained in repetitive responses at the rate of 5 stimulations per second with atropine 1:2,000 after 15, 30, 50, 70 and 90 minutes of alkaloid action. Fig. 11 (2) shows on the same scale the (per cent.) decreases for two repetitive responses each of hyoscine, tigloidine and lasiocarpine. The action potential drops from response to response, quickly at first and more slowly later on. The curves obtained in the different repetitive bursts and with the different alkaloids are very similar; the start of the curve on the graph is, of course, determined by the size of the action potential of the first response; its course, however, appears to follow an identical pattern in all cases. Compared with the decrease of action potential as observed *with the time of alkaloid action* in the depressant effect, the curves are more rounded; but they do not seem to be essentially different from the curves of decrease as observed with isolated responses obtained with delayed-recovery drugs *with the time of alkaloid action.*

Time of conduction. The time of conduction increases steadily on consecutive stimulations. Figs. 10 (2) and 11 (2) show the changes for the same experiments as reported for the action potential. It will be seen that in Fig. 10 (2) the increases for the first repetitive stimulation, after 15 minutes of alkaloid action, stand apart from the fairly parallel bundle of the later ones: the delayed-recovery effect was not yet fully developed. This fact does *not* show in the corresponding curve of the action potential; the observation was repeatedly made with very early repetitive responses. Once the effect is fully developed the curves are very similar within the same experiment; in different experiments they may differ widely. Lasiocarpine, 45 and 90 minutes (Fig. 11 (2)) shows almost no difference, while tigloidine, 35 and 60 minutes, shows about 25 p.c. distance between the curves. The absolute increase in time of conduction may reach 200 and 350 p.c.; such increases are not observed in the merely depressant effect; furthermore the course of the curves differs from that of the decrease of action potential in so far as the curves are *almost rectilinear at the beginning* and more rounded later on; the radius of curvature increases; in later responses the curves may be rectilinear throughout the number of observable responses. The curves have a characteristic and constant pattern within the same experiment, but the pattern is different from that in which the action potential decreases, the main difference being the almost rectilinear start.

Relative changes of action potential and time of conduction. This relation is illustrated in Figs. 10 (3) and 11 (3). As could be expected the more steady increase in time of conduction determines the shape of the curve. In Fig. 10 (3), atropine, the first response, is out of line, owing to the lower increase of time of conduction in the not yet fully developed effect; the other curves seem, if somewhat untidily, rather to coincide. From Fig. 11 (3), however, it is seen that the onset of the curves depends on the changes of action potential and time of conduction *between* the various repetitive bursts; if the time of conduction increases only slightly as in the experiment with lasiocarpine, the curves of the later repetitive responses are placed below the previous ones; if the time of conduction increases more, they may coincide or even overlap. The course, not the onset, of the curves is characteristic. While in the depressant effect the curves (Fig. 6 (2)) fall within a rectangle of 45°, straight or slightly *convex* with regard to a centre of curvature somewhere near the crossing of the axes, the curves of the delayed-recovery effect fall outside such a triangle and are concave with regard to the same location of reference. This is considered to be a characteristic difference between the changes in the depressant effect with time of alkaloid action and those in the delayed-recovery effect within a repetitive burst; its representation in the graph is of course, dependent on the

scale of plotting; the direction of curvature is, however, not affected by linear variation of the scale. It is further seen that despite the considerable differences in the rates of increase in time of conduction (Fig. 11 (2)) the resultants of the relative changes of time of conduction *and* action potential are about the same. It is considered that these relations follow a distinct pattern of their own; the difference to the depressant effect may tentatively be formulated as: *in the depressant effect the increase in time of conduction lags behind the decrease of action potential, in the delayed-recovery effect it exceeds it.*

Tension. With the apparatus available it was not possible to measure the change of tension during the consecutive responses of a repetitive stimulation. It can only be said that the tension becomes unobservable at about the same time as the diphasic wave, and that it seems to drop parallel with the action potential.

The threshold of stimulation appears to increase slightly during a repetitive stimulation. The recovery is, however, too quick to allow exact measurement with the apparatus used.

Recovery. Despite the almost complete disappearance of the response after a repetitive burst of 10–15 stimuli a short rest restores the reactivity of the muscle to its initial level. The length of the time needed for complete recovery depends on the intensity and duration of the alkaloid action as well as on the intensity of the previous stimulation. At the early states of alkaloid action it amounts to a few seconds only, later on, particularly after strong stimulation it may be one minute or more before a response can be repeated more or less identical to the previous one. Any stimulation before complete recovery leads to weaker responses and quicker exhaustion; it will appear that the recovery is delayed but otherwise unaltered. No indication was found to suggest that prolonged stimulation would lead to a permanent damage.

Onset of the effect. No evidence can be presented which would suggest a connection between the onset of the fully developed delayed-recovery effect and the time of alkaloid action, the changes of action potential or time of conduction in isolated responses, previous stimulations or any other ascertainable data. It will appear that the rate at which the effect develops is dependent on the specific nature of the alkaloid only. Tigloidine and lasiocarpine show the fully developed effect very early when the original values of action potential and time of conduction have barely changed; apo-atropine leads to a rapid drop of the values in isolated responses and quick abolition of all response, while the delayed-recovery effect develops slowly and is never present to the extent in which it is shown by the former; atropine and hyoscine are intermediate.

Constancy of the effect. Once the effect is fully developed, its intensity appears to be persistent throughout the experiment; the rate at which in successive responses the action potential drops, or the time of conduction increases is not noticeably altered in later responses. It will appear that the effect reaches a maximum intensity early in the experiment and maintains it throughout it; it does certainly not alter parallel to the changes which are shown in isolated responses. Nor is there any indication that its intensity is affected by previous stimulation if time for complete recovery is allowed; the responses seem to be the same whether the muscle is much stimulated or only sparingly.

The shape of the wave. The diphasic wave in the later responses quickly loses the characteristic shape of the first response; it becomes rounded and flat. Even the angularity of the first spike disappears and the later responses of a repetitive stimulation present themselves as slight, almost symmetrical undulations. The delayed return to zero level is more marked earlier in the experiment only because there are still angles and almost straight slopes to contrast it.

Duration of the wave. The distance between the two spikes or, later on, between the two crests of the diphasic response is not essentially altered; it is certainly not stretched in proportion to the increase in time of conduction.

These observations may be summarized as follows: under the influence of a delayed-recovery drug the muscle reaches within a short repetitive stimulation the same complete abolition of responses which the depressant drugs produce within an hour or more. This quick "fatigue" is, however, short-lived and a rest of a few seconds or more, according to the intensity of drug action, restores the muscle to its previous level of reactivity. The pathway in which abolition of responses is achieved is different in the two effects:

Ratio of increase of time of conduction to decrease of action potential. In the depressant effect the increase in time of conduction lags behind the decrease of action potential, in the delayed-recovery effect it exceeds it.

Shape of the wave. In the depressant effect the wave retains its angularity almost to the last, barely noticeable responses; in the delayed-recovery effect the return from the lower spike to zero level is delayed as soon as the effect becomes noticeable; in later responses of a repetitive stimulation or later on in the action of the drug the whole wave becomes progressively rounded; spikes and angles disappear.

The duration of the wave appears to be only slightly altered in either of the effects.

The two effects do not appear to be connected; in both cases the muscle reaches a final state when it is no longer reactive; in the case of a delayed-recovery drug it reaches this state just as quickly whether it is stimulated and forced to recover or not. The fact of exhaustive stimulation does not appear to affect the muscle in any way, except during the period of recovery. The delayed-recovery effect appears to be a temporary phenomenon in addition to the normal depressant effect of the alkaloid.

Discussion of the Chemical Relations.

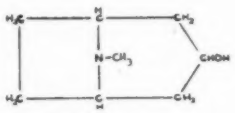
As far as the tropine and heliotridine esters were investigated it was found that those alkaloids which are esterified with *saturated* acids cause only the depressant effect, while those esterified with acids containing either a benzene ring or aliphatic *unsaturation* cause the effect of delayed recovery. A list of the alkaloids follows with references to the constitution of the rarer compounds.

Alkaloid	*Base*	*Esterifying Acid*
delayed-recovery effect:		
Atropine-hyoscyamine	tropine	tropic acid
Apo-atropine	tropine	atropic acid
Hyoscine	scopine	tropic acid
tigloidine (1)	pseudotropine	tiglic acid
lasiocarpine (2)	heliotridine	angelic (and lasiocarpic) acids.

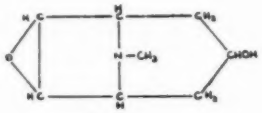
depressant effect only:

Valeroidine (3)	hydroxytropine	iso-valeric acid
Valtropine (4)	tropine	iso-valeric acid
Poroidine (5)	nortropine	methyl-butyric acid
iso-poroidine (5)	nortropine	iso-valeric acid
iso-heliotrine (6)	heliotridine	heliotric acid

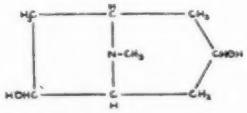
The basic and acidic components of the esterified alkaloids which were investigated.



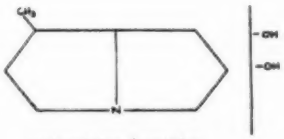
tropine and pseudotropine



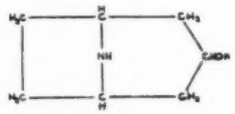
ecopine



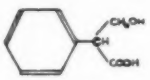
hydroxytropine



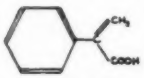
heliotridine (contains one double bond and two alcoholic groups of unknown position)



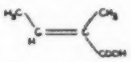
nortropine



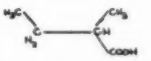
tropic acid



atropic acid



tiglic acid and angelic acid



alpha-methyl-butyric acid

iso-valeric acid

lasiocarpic acid $C_8H_{16}O_5$ and heliotric acid $C_8H_{16}O_4$ are saturated acids of unknown constitution.

Fig. 12.

The comparison of the formulae leads to a few interesting suggestions:

1. It will appear that, as far as the tropine bases are concerned, substitution in the nucleus other than the nitrogen-ester-link constellation has little effect on the action observed. The oxygen substituted scopine if esterified with tropic acid has an effect indistinguishable from that of atropine or hyoscyamine, while the oxygen substituted hydroxy-tropine if esterified with the saturated iso-valeric acid shows the depressant effect only and is indistinguishable from valtropine which is tropine esterified with the same acid.

2. The secondary base nor-tropine esterified with iso-valeric acid is possibly slightly more active in its depressant effect on the muscle than tropine or hydroxytropine esterified with the same acid; it appears that the tertiary nitrogen is not necessary for the effect.

3. Furthermore, it would appear that the optical configuration has no influence on the the effects observed. Tigloidine contains pseudotropine and when esterified with the unsaturated tiglic acid, produces the same effect of delayed recovery as the optical isomer tropine esterified with tropic or atropic acids; tiglic acid in tigloidine appears to be responsible for the effect of delayed recovery, just as its optical isomer angelic acid is in lasiocarpine.

The grouping described is present in several anaesthetics and related compounds; their effect on the isolated sartorius of the toad is similar but not always identical to that of the alkaloids described. These compounds contain other reactive groupings besides the nitrogen-2 or 3 carbon-ester-link; a series of them is under investigation. Fig. 13 gives the formula of pantocaine, the characteristic grouping being drawn in thick lines and the dotted lines indicating the ring-closure of the aliphatic chain to the heliotridine structure.

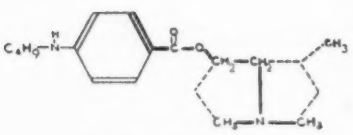


Fig. 13. Pantocaine.

The constitution of heliotridine and of the saturated aliphatic acids heliotric and lasiocarpic acid is not yet completely established; the base contains one aliphatic double bond and two alcoholic groups, the acids are probably branched-chain acids containing alcoholic and methoxygroups. The bases were chosen for investigation because they are esterified bases with a nucleus which presents a slight variation of the tropine nucleus. On the previously obtained results with the tropine alkaloids it was expected that the free unesterified alcoholic group of iso-heliotrine would have as little influence on the effect as the free alcoholic group in valeroidine and that the oxygen substitution of the saturated acids would likewise be without effect; on the other hand it was expected that the unsaturated angelic acid of lasiocarpine would cause an effect similar to that of tiglic acid in tigloidine. Both these expectations were verified by experiment and for this reason the alkaloids were included in the present publication although their exact constitu-

tion is unknown. It will appear that within the group of alkaloids examined the effect of delayed recovery is conditioned essentially by the secondary or tertiary nitrogen atom linked by two or three carbon atoms to a carboxyl group esterified with an aromatic or unsaturated aliphatic acid.

One further observation presents itself: all the alkaloids examined can be regarded as derivatives of a secondary or tertiary homologue of acetylcholine, arrived at by ring-closure and by esterification with a different acid. The basic constituents would be, to consider the tertiary bases only, dimethylamino-ethanol or dimethylamino-propanol:

The dotted lines (Fig. 14) present the ring-closure to give the tropine and heliotridine nucleus. In the latter case the position of the alcoholic group is arbitrary, but it will be seen that any other of the four possible positions (marked with asterisks) leads to the same result: the configuration is that of an aminoethanol if the distance between nitrogen and ester link is passed in one direction and that of an aminopropanol if it is passed in the other direction round the ring.

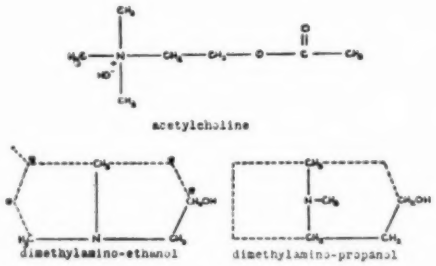


Fig. 14.

At the present state of the investigation it is not intended to draw any far-reaching conclusions from this observation and to enter into the discussion of the arguments for or against an acetylcholine mechanism taking part in conduction or response of the muscle. In the case of yohimbine, examined by Shaw and co-workers, the acidic group forms part of the nitrogen-containing ring system of the alkaloid molecule and is esterified with a simple methyl group; without further evidence it would be far-fetched to trace an acetylcholine homologue into the yohimbine structure; the findings with the tropine and heliotridine bases may present an accidental and unessential analogy, however suggestive they may appear.

The investigation is being continued.

SUMMARY.

Naturally occurring alkaloids of the tropine and heliotridine group were investigated in their effect on the diphasic response on the sartorius of the toad *Bufo marinus*. Two different effects were noted:

The "depressant effect" is characterized by a gradual fall of the action potential to final abolition together with a 50–100 p.c. increase in the time of conduction. During repetitive stimulation the shape and amplitude of the electric response and the time of conduction are unaltered. This effect was obtained with valeroidine, poroidine, iso-poroidine, iso-valeryl-tropine and iso-heliotrine.

The isolated single stimulation under the action of alkaloids showing the "delayed-recovery effect" resembles in its shape and time of conduction those produced in the depressant effect except that the return of the diphasic wave to zero level appears to be delayed. The characteristics of the delayed-recovery effect are seen on repeated stimulation; the action potentials of the second and following responses of a repetitive burst decrease quickly, 10–15 stimulations at the rate of 5–7 per second leading to complete abolition; the time of conduction increases by 100 to 150 p.c. and, depending on the duration and intensity of the alkaloid action, the muscle needs from several seconds to several minutes for complete recovery to occur. This effect was obtained with atropine, apo-atropine, hyoscine, tigloidine and lasiocarpine.

It was found that all alkaloids causing the delayed-recovery effect are esterified with an acid containing either a benzene nucleus or aliphatic unsaturation, while all those showing the depressant effect are esterified with saturated acids.

Attention is drawn to the fact that the alkaloids in question can be regarded as substituted tertiary homologues of acetylcholine, but no interpretation of the effects was attempted at the present state of the investigation.

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THE TOXICITY OF POTASSIUM CHLORIDE AND OF EXTRACTS OF *PSALLIOTA XANTHODERMA* FOR MICE AND RABBITS

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The sporophores of the edible mushroom *Psalliota xanthoderma,* which have been shown (Atkinson, 1946) to exert a powerful antibacterial action, have yielded dried aqueous extracts which contain a high concentration of potassium chloride. Accordingly, in view of the pronounced physiological action (Miller and Darrow, 1940) of the potassium ion in the animal body, it was considered worthwhile to investigate the toxicity of dried aqueous extracts of *Psalliota xanthoderma.*

The experimental animals used were mice and rabbits and the response of these animals to parenteral injection of *Psalliota xanthoderma* extract was compared with that observed when potassium chloride was injected.

It has been shown in this paper that the toxic effect of the mushroom extract upon these animals can be attributed to its potassium content.

EXPERIMENTAL.

Materials and Methods.

The potassium chloride used was British Drug Houses AR quality and was dissolved in distilled water to give a 20 p.c. solution. In the case of the lower doses, where the volume would have been inconveniently small for injection, the 20 p.c. solution was diluted to 10 p.c.

The *Psalliota xanthoderma* solutions used were prepared from various dried extracts by dissolving in distilled water. The dried extracts had been prepared by mincing the sporophores, grinding the minced material with water, expressing the juice, filtering free of cellular debris and foreign material and finally drying from the frozen state.

Potassium was estimated in the *Psalliota xanthoderma* extracts and in the specimens of serum from the experimental animals by the sodium-cobalti-nitrite method of Kramer and Tisdall (1921).

The Toxicity of Potassium Chloride.

Mice: Intraperitoneal Injection.

Twenty-five mice, each weighing approximately 20 gm., were randomized into five equal groups and were given intraperitoneal injections of potassium chloride. The doses given to

each group formed a graded series from 0·715 gm./kg. body weight to 2·74 gm./kg. in 40 p.c. increments and the results obtained were subjected to statistical analysis to determine the LD 50. The method used was the method of probits as described by Fisher and Yates (1938).

The results and protocol are shown in Table 1.

TABLE 1.

Toxicity for mice of potassium chloride: intraperitoneal injection.

Mouse No.	Weight gm.	Dose level gm./kg.	Vol. injected ml.	Death time mins.
1	20·0		*0·14	S
2	21·0		0·15	S
3	19·5	0·715	0·14	S
4	20·0		0·14	25½
5	20·0		0·14	S
6	20·0		0·10	24
7	23·0		0·115	S
8	19·5	1·0	0·097	22
9	20·0		0·10	S
10	22·5		0·113	1
11	22·0		0·154	S
12	22·0		0·154	S
13	18·0	1·4	0·126	S
14	19·0		0·133	63
15	21·0		0·147	S
16	20·5		0·20	S
17	20·0		0·196	11
18	21·0	1·96	0·21	S
19	18·0		0·176	8
20	16·0		0·157	7
21	19·0		0·26	7
22	21·0		0·29	113
23	21·0	2·74	0·29	10
24	21·0		0·29	6
25	16·5		0·25	4
26	22·5		*0·62	S
27	21·0		0·58	5½
28	22·0		0·60	6
29	20·5	2·74	0·56	4
30	24·0		0·66	5½
31	24·5		0·67	5
32	23·0		0·63	4½
33	22·5		0·62	4
34	24·0		0·66	207

* 10 p.c. aqueous solution used. S survived.

The mice which died after the injection showed the signs and symptoms of potassium intoxication. Immediately after injection they showed a disinclination to move about and they remained quiet and huddled together. General muscular weakness then became apparent. The rear legs were particularly affected and the mice were unable to use them. When they were disturbed, the mice moved with great difficulty, dragging the paralysed rear limbs behind them. Dyspnoea and convulsions preceded death, which usually occurred soon after the onset of the convulsions. At post mortem the heart was seen to be in diastole.

The LD 50 of potassium chloride for mice injected intraperitoneally was calculated by the method of probits as 1·3 gm./kg. body weight.

Rabbits: Intravenous Injection.

Six rabbits received injections of potassium chloride into an ear vein. Three rabbits received no injection of potassium chloride and were used as normal controls to establish the normal serum potassium level. Two were killed by air embolism, 25 ml. of air being injected into an ear vein, while the third was killed by concussion.

Samples of heart blood were obtained with a needle and syringe by direct puncture, after exposing the heart by opening the thoracic cavity within three minutes of death. The serum was separated by immediate centrifugation, great care being taken to avoid haemolysis, and was kept in the cold room at 4° C. until the potassium content could be estimated. Table 2 shows the results and protocol.

TABLE 2.

Toxicity for rabbits of potassium chloride: intravenous injection.

Rabbit No.	Weight kg.	Dose level mg./kg.	Vol. injected ml.	Death time mins.	Serum potassium mg./100 ml.	Mean
1	2·71	40	1·1	S	—	
2	2·80	56·6	1·58	S	—	
3	2·71	80	2·17	1¼	92	
4	2·36	80	1·89	1½	100	117 ± 29·5
5	2·48	80	1·98	1¼	167	
6	2·31	80	1·85	1¼	109	
Controls						
1	2·92	Nil	Nil	killed AE	31	
2	2·86	Nil	Nil	killed AE	35	30 ± 4·5
3	2·50	Nil	Nil	killed C	24	

S Survived. — Not estimated. Killed AE: Killed by air embolism. Killed C: Killed by concussion.

Deaths occurred in from 1¼–1½ minutes after the completion of the injection; convulsions preceded death in two out of the four rabbits and profound dyspnoea was observed in all cases.

The two rabbits which received 40 mg./kg. and 56·6 mg./kg. were both dyspnoeic and were disinclined to move at 1–1½ minutes after injection but they gradually recovered and were completely recovered within four hours.

Toxicity of Extracts of Psalliota xanthoderma.

Mice: Intraperitoneal Injection.

Three separate batches of dried aqueous extract of *Psalliota xanthoderma* were taken and after analysing to determine their potassium content were dissolved in distilled water to give a dose level, expressed as potassium chloride, of 2·74 gm./kg. body weight.

Twenty-nine mice were used in this experiment and the results and protocol are shown in Table 3.

The signs and symptoms observed in the mice injected at the dose levels of *Psalliota xanthoderma* extract corresponding to 2·7 gm./kg. potassium chloride were exactly the same as those observed in the mice which were injected with potassium chloride alone at the same level.

TABLE 3.

Toxicity for mice of solution of dried extract of Psalliota xanthoderma: *intraperitoneal injection.*

Mushroom batch No.	Mouse No.	Weight gm.	Vol. injected ml.	Dose level gm./kg.		Death time mins.
				Psalliota extract	expressed as KCl	
2 B	1	21·5	0·56			4
Dry material contained 16·6 p.c. KCl	2	19·0	0·49			5
	3	21·0	0·55			11
	4	21·0	0·55			8
	5	23·0	0·60			30
	6	20·0	0·52			6½
	7	18·5	0·48			5½
	8	19·5	0·50	16·11	2·69	3½
	9	20·0	0·52			6
	10	22·0	0·57			51
	11	16·5	0·43			5½
	12	23·0	0·60			6
	13	23·5	0·61			6½
	14	21·0	0·55			5
	15	19·0	0·49			7
21 I	1	20·0	0·49			250
Dry material contained 17·2 p.c. KCl	2	20·0	0·49			6
	3	22·0	0·54			5½
	4	20·0	0·49			65
	5	20·0	0·49	15·95	2·74	5
	6	20·0	0·49			43
	7	20·5	0·50			78
	8	19·0	0·47			3½
	9	18·0	0·44			75
24 G	1	20·0	0·63			123
Dry material contained 13·3 p.c. KCl	2	18·5	0·59			196
	3	21·0	0·67	20·66	2·75	5½
	4	15·0	0·48			6½
	5	18·0	0·57			5

Rabbits: Intravenous Injection.

Four rabbits were injected intravenously with an aqueous solution of a dried extract of *Psalliota xanthoderma*. Table 4 shows the protocol and results obtained.

TABLE 4.

Toxicity for rabbits of solution of dried extract of Psalliota xanthoderma: *intravenous injection.*

Rabbit No.	Weight kg.	Vol. injected ml.	Dose level mg./kg.		Death time mins.	Serum Potassium mg./100 ml.	Mean
			Psalliota extract	expressed as KCl			
1	2·80	2·23	400	50	S	—	
2	2·66	3·00	566	71	1¼	116	
3	2·80	3·16	566	71	1¾	116	111 ± 6·4
4	1·88	2·12	566	71	1¾	100	

S Survived. — Not estimated.

Deaths occurred in from 1¼–1¾ minutes after injection of 71 mg./kg. expressed as potassium chloride; convulsions and profound dyspnoea were observed in the three rabbits from the moment of injection to the time of death. The rabbit which received *Psalliota xantho-*

derma extract at a dose level corresponding to 50 mg./kg. potassium chloride, became very quiet and dyspnoeic at 1½ minutes, but was completely recovered within four hours. The similarity of the signs and symptoms with those observed after injection of potassium chloride alone, indicate that the toxicity of *Psalliota xanthoderma* extracts for rabbits after intravenous injection can be attributed to the potassium chloride content.

DISCUSSION.

The action of the potassium ion upon heart muscle is to reduce its contractility and favour its relaxation. This effect is antagonized by the calcium ion but, if the potassium ion is in excess, more and more of the cardiac cycle is occupied by diastole and the heart ultimately comes to rest in the completely relaxed state—a condition known as potassium inhibition (Best and Taylor, 1945). In the experiments reported in this paper the heart stopped in diastole in all cases in both the potassium chloride and the *Psalliota xanthoderma* groups.

Miller and Darrow (1940) record a series of experiments on the toxicity of potassium chloride injected intraperitoneally into adult male rats. They found the normal serum potassium level of 13 rats to be 16 mg./100 ml. with a standard deviation of 0·9 mg. and stated that, in their experience, death occurred or was about to occur when the serum potassium level rose to 80 mg./100 ml.

In the present paper the figures obtained for the serum potassium level in rabbits have been 30 mg./100 ml. with a standard deviation of 4·5 mg. for normal rabbits, 117 mg./100 ml. with a standard deviation of 29·5 mg. for rabbits injected with potassium chloride and 111 mg./100 ml. with a standard deviation of 6·4 mg. for rabbits injected with an extract of *Psalliota xanthoderma*. The toxicity of *Psalliota xanthoderma* extracts at the concentration injected into rabbits by the intravenous route is thus due to the potassium chloride which they contain.

Miller and Darrow (1940) further state, that if death does not occur in rats receiving intraperitoneal injections of potassium chloride within 50–66 minutes of injection, then recovery usually occurs. In the present paper, of the 13 mice which died after receiving intraperitoneal injections of potassium chloride, 2 (15·4 p.c.) survived for longer than 60 minutes, while of the 29 mice which died after receiving *Psalliota xanthoderma* injections, 6 (20·7 p.c.) survived for longer than 60 minutes. The observed toxic effects of *Psalliota xanthoderma* extracts, injected intraperitoneally into mice at a dose level corresponding to 2·7 gm./kg. of potassium chloride, appear to be the same as that observed when potassium chloride alone is injected at the same dose level.

The possibility of a slower acting toxic substance cannot be excluded however, because the rapid killing effect of the potassium ion would mask it.

SUMMARY.

The parenteral injection of potassium chloride into mice and rabbits produces the typical picture of potassium inhibition of heart action.

Psalliota xanthoderma extracts show the same action due to their content of potassium chloride.

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